PROTECTIVE STRATEGIES AGAINST MYOCARDIAL ISCHEMIA-REPERFUSION INJURY: EXERCISE AND NUTRITIONAL ANTIOXIDANTS

By

KARYN L. HAMILTON

A DISSERTATION PROPOSAL PRESENTED TO THE GRADUATE SCHOOL OF
THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2000

ACKNOWLEDGMENTS

This project could not have been completed without the cooperation of many individuals. Above all, I would like to convey my most heartfelt appreciation to my committee chair and mentor, Dr. Scott Powers, for his unending support and encouragement throughout my doctoral program at the University of Florida. His enthusiasm, availability, and dedication to his students and the pursuit of scientific understanding remain unmatched by anyone with whom I have come in contact. I will always be one of his greatest admirers.

I would also like to thank my doctoral committee, Drs. Stephen Dodd, Christiaan Leeuwenburgh, and Ralph Dawson, for their patience and expertise throughout these experiments. Additionally, I would like to thank my two student mentors, Jeff Coombes and Haydar Demirel. Without their guidance during my first years in the laboratory, the road to finishing this Ph.D. would have been much rockier and far less enjoyable. Many of my coworkers also contributed to the success of this project. These individuals include Shannon Lennon, Tracey Phillips, Andrea Hess, April Childs, Thomas Koesterer, Wes Smith, and Andy Shanely. A very special thank you goes to Jessica Staib, without whom I could not have completed this project. Finally, I would like to express my gratitude to my mother who has always been my greatest source of support, encouragement, and strength. A portion of this degree belongs to her.

This work was supported by a grant from the American Heart Association - Florida Affiliate.

TABLE OF CONTENTS

page

ACKNOWLEDGMENTS ii

TABLE OF CONTENTS iii

LIST OF TABLES v

LIST OF FIGURES vi

CHAPTERS
INTRODUCTION 1
Significance 2
Specific Aims 3
Hypothesis Justification 4

2. REVIEW OF RELATED LITERATURE 7
  Introduction 7
  Myocardial Ischemia-Reperfusion Injury 8
  Reactive Oxygen Species and Ischemia-Reperfusion Injury 9
    Sources of reactive oxygen species 9
    Myocardial lipid peroxidation 11
    Myocardial protein oxidation 11
  Antioxidant Defense Mechanisms 12
    Vitamin E 13
    Vitamin C 15
    Glutathione 16
    Carotenoids 17
    Flavonoids 18
    Ubiquinones 19
  Heat Shock Proteins: An Overview 21
    Overview of HSP families 22
    Biochemical roles of 70-kDa HSPs 24
    Regulation of HSP induction 25
    HSP72 and cytoprotection 26
    HSP72 and protection against myocardial ischemia-reperfusion injury 27
    Exercise-induced HSP72 expression and myocardial protection 27
  Summary 29

3. METHODS AND PROCEDURES 31
Animals and Experimental Design....................................31
Exercise Training Protocol....................................33
In Vivo Myocardial Ischemia-Reperfusion Protocol....................................33
Measurement of Cardiac Contractile Function 34
Assessment of Myocardial Infarction Size....................................35
Tissue Removal, Storage, and Preparation....................................36
Tissue removal....................................36
Preparation of protein extract 36
Polyacrylamide Gel Electrophoresis and Western Blotting....................................37
Assessment of Myocardial Antioxidant Status 37
Biochemical assessment of antioxidant enzyme activity....................................37
Total antioxidant capacity assay....................................38
Biochemical Indicators of Oxidative Stress....................................39
Lipid peroxidation measurements....................................39
Protein oxidation....................................40
Model Justification....................................41
Statistical Analyses 42

4. RESULTS....................................43
Overview of Experimental Findings 43
Antioxidant Intake....................................43
Myocardial Contractile Function 44
Myocardial Infarction....................................46
Myocardial HSP72 Accumulation 47
Total Antioxidant Capacity 49
Myocardial Antioxidant Enzyme Activity....................................49
Lipid Peroxidation....................................51
Protein Oxidation 53

5. DISCUSSION....................................55
Overview of Principal Findings 55
Exercise-Associated Cardioprotection and HSP72....................................56
Exercise and Antioxidants Attenuate Infarction Size 57
Antioxidant Supplementation and Myocardial Antioxidant Capacity 58
Myocardial Lipid Peroxidation 58
Myocardial Protein Oxidation....................................60
Possible Mechanisms of Exercise-Associated Cardioprotection 62
Summary and Conclusions....................................64

REFERENCES 66

BIOGRAPHICAL SKETCH....................................91

LIST OF TABLES

Table Page

1. Antioxidant diet composition....31

2. Food and antioxidant intake....44

3. Summary of the effects of exercise training and antioxidant supplementation on endogenous antioxidant enzyme activity....51

LIST OF FIGURES

Figure Page

1. Experimental design……………………………………………………………32
2. Left ventricular developed pressure……………………………………………45
3. Risk area expressed as a percentage of the heart………………………………46
4. Infarction are expressed as a percentage of the area at risk…………………47
5. Representative heart sections stained for infarction following long-term I-R………47
6. Left ventricular HSP72…………………………………………………………48
7. Assessment of total antioxidant status…………………………………………50
8. Left ventricular lipid peroxidation……………………………………………52
9. Left ventricular protein thiol concentration…………………………………53
10. Left ventricular protein carbonyl concentration………………………………54

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

PROTECTIVE STRATEGIES AGAINST MYOCARDIAL ISCHEMIA-REPERFUSION INJURY: EXERCISE AND NUTRITIONAL ANTIOXIDANTS

By

Karyn L. Hamilton

December, 2000

Chairman: Scott K. Powers, Ed.D., Ph.D.
Major Department: Exercise and Sport Sciences

Numerous investigations indicate that increasing myocardial heat shock protein (HSP) content, as well as enhancing myocardial antioxidant capacity, is associated with an attenuation of damage resulting from ischemia-reperfusion (I-R). Therefore, developing strategies to increase myocardial antioxidant capacity and/or HSPs is important for minimizing ischemic injury. We have recently shown that exercise-training or antioxidant-enriched diets can independently decrease I-R-induced myocardial injury *in vivo*. Although, exercise and antioxidants can both protect the heart against I-R-induced injury, the interaction between these two interventions has not been investigated. Because exercise and antioxidants may provide cardioprotection via different mechanisms, it is possible that the combination of the two could provide additive protection. Alternatively, because oxidants may play a vital role in the induction of HSPs, antioxidant supplementation may attenuate exercise-related cardioprotection. These experiments examined the individual and combined effects of exercise-training and antioxidants on responses to *in vivo* myocardial I-R in adult rats. Animals were randomized to receive either an antioxidant-supplemented diet or a control diet. Following 6 weeks of feeding, rats were assigned to either an exercise-training group (five days treadmill running) or a sedentary control group. Finally, rats were randomized to one of three surgical treatments: (1) short-term I-R; (2) long-term I-R; or (3) sham. Antioxidant supplementation attenuated the exercise-associated accumulation of a 72 kDa HSP. Nonetheless, during short-term I/R, exercised groups demonstrated improved ($p<0.05$) cardiac contractile performance regardless of diet. Compared to control diet/sedentary animals, long-term I/R resulted in a smaller infarction in all groups. The combination of exercise and antioxidants resulted in the greatest protection against infarction. Antioxidant supplementation, regardless of training, protected the myocardium from lipid peroxidation and protein oxidation. While both exercise and antioxidants appear to provide cardioprotection during I-R insults, no synergistic or antagonistic relationship was evident.

CHAPTER 1
INTRODUCTION

Cardiac injury resulting from ischemia-reperfusion (I-R) is an important clinical problem with ischemia-induced pathological processes, such as heart disease, constituting the single greatest cause of death in this country. Hence, developing interventions that can protect the myocardium from I-R associated damage is an important research endeavor.

Recent investigations strongly suggest that heat shock proteins (HSPs) of the 70 kDa family are important in protecting cells from ischemia-reperfusion (I-R) induced injury. It has been demonstrated that endurance exercise training elevates myocardial HSP72 in young adult rats. Importantly, this exercise-induced increase in HSP72 is associated with cardioprotection during I-R. While the specific exercise-induced cellular changes responsible for signaling an up-regulation of HSPs remain unclear, an increased production of reactive oxygen species (ROS) has been implicated.

While ROS may serve as a necessary signal for induction of protective HSPs, it is clear that ROS contribute to I-R induced injury to the myocardium. Enhanced antioxidant defenses can provide protection against ROS-mediated injury. Recent investigations have shown that increasing myocardial antioxidant capacity attenuates I-R-mediated oxidative damage. However, little is known about the effects of antioxidant supplementation on induction of myocardial HSPs. If oxidative stress is, indeed, a key signal for inducing HSPs, supplementation with antioxidant nutrients may result in an attenuation of the HSP response to exercise stress. Therefore, the aims of this study were as follows: (1) to investigate the individual and combined effects of short term exercise training and supplementation with nutritional antioxidants on the expression of HSPs in the 70 kDa family and (2) to examine the effect of short term exercise training and supplementation with nutritional antioxidants on protection against myocardial ischemia-reperfusion injury.

Significance

Heart disease is the most prevalent chronic disease in the United States, accounting for approximately 1.5 million myocardial infarctions per year (206). Therefore, strategies for protecting the myocardium against ischemic insults are clearly needed. Exercise training, for example, has been shown to provide cardioprotection during ischemia-reperfusion (I-R), perhaps in association with induction of heat shock proteins (HSPs). Another potential strategy for providing enhanced defenses against I-R induced cardiomyocyte injury is via supplementation with nutritional antioxidants (8,69,72,74, 86-87,124,128,158,166,201,218). The mechanisms by which exercise and nutritional antioxidants provide myocardial protection are not fully understood. Whether the interaction between antioxidant supplementation and exercise training on providing protection during myocardial I-R is synergistic versus antagonistic remains unknown. The results of these experiments help elucidate the impact of exercise and nutritional antioxidant interventions on the extent of I-R induced contractile dysfunction, myocardial infarction, and oxidative injury, as well as the ability of myocardial cells to up-regulate HSP expression. Collectively, these experiments provide important progress toward developing successful, practical strategies to increase myocardial defenses against I-R injury.

Specific Aims

Exercise training and antioxidant supplementation are interventions that have been independently shown to provide protection against myocardial I-R damage (24,43-44,52,54,93-94,198,233). The overall purpose of this study was to investigate the individual and combined effects of short-term exercise training and supplementation with nutritional antioxidants on myocardial protection during I-R. Specifically, the following questions were addressed.

Question 1

What is the effect of short-term exercise training and nutritional antioxidant supplementation on induction of 70 kDa HSPs compared to exercise training alone?

Hypothesis 1

Induction of 70 kDa HSPs will be attenuated following the combined intervention of exercise training and antioxidant supplementation compared to exercise training alone.

Question 2

What is the effect of the combined intervention using short-term exercise training and nutritional antioxidant supplementation on myocardial contractile performance during *in vivo* ischemia-reperfusion compared to exercise training alone and antioxidant supplementation alone?

Hypothesis 2

(2a) Myocardial contractile performance following short-term exercise training and antioxidant supplementation will be enhanced compared to control animals but suppressed compared with animals that were exercise trained only. (2b) Myocardial contractile performance following antioxidant supplementation alone will be enhanced compared to control but to a lesser extent than with exercise training alone.

Question 3

What is the effect of the combined intervention using short-term exercise training and nutritional antioxidant supplementation on protection against myocardial infarction following *in vivo* ischemia-reperfusion compared to exercise training alone and antioxidant supplementation alone?

Hypothesis 3

(3a) Protection against myocardial infarction following short-term exercise training and antioxidant supplementation will be enhanced compared to control animals but suppressed compared with animals that were exercise trained only. (3b) Protection against myocardial infarction following antioxidant supplementation alone will be enhanced compared to control but to a lesser extent than with exercise training alone.

Hypothesis Justification

It has been previously demonstrated that exercise training results in improved contractile performance during myocardial I-R in rats (24,54,198,236). These exercise-induced changes are correlated with the induction of myocardial HSP72 (54,56,148-149,198). While the specific exercise-induced cellular changes responsible for signaling an up-regulation of HSPs remain unclear, an increased production of reactive oxygen species (ROS) has been implicated (63,139,170-172,248-249). It is clear that cytotoxic ROS contribute to I-R induced injury in the heart and that supplementation with antioxidants can provide protection against ROS-mediated injury. However, little is known about the effects of antioxidant supplementation on myocardial expression of HSPs. Because it is likely that exercise training and dietary antioxidant supplementation provide cellular protection against I-R injury via different mechanisms, it is possible that their combined effects could provide additive protection against I-R-induced oxidative stress. However, if oxidative stress is, indeed, a key signal for inducing HSPs, supplementation with antioxidant nutrients may result in an attenuation of the HSP response to exercise stress. Thus, in the proposed experiments, the hypotheses were based on the notion that the combination of antioxidant supplementation with exercise training will result in an attenuation of HSP72 induction and, therefore, an attenuation of the cardioprotection associated with exercise training alone (84,239).

In addition to the above, we hypothesize that antioxidant supplementation will result in improved contractile performance and protection against oxidative damage and infarction compared to control. That is, supplementation with a combination of potent aqueous- and lipid-phase antioxidants will be associated with a decrease in myocardial lipid peroxidation and protein oxidation and this will result in an attenuation of myocardial infarction size. These hypotheses were based on the known ROS-quenching abilities of antioxidants and the finding that both lipid peroxidation and cardiac protein oxidation occurs during I-R (37-40,88-89,99,127,185). It is speculated that protection from lipid peroxidation will be primarily due to elevated myocardial vitamin E, β-carotene, and coenzyme Q_{10} because they reside in the lipid phase of membranes (8,66,69,72,74,86-87,124,128,156,166,169,174,201,213,218,225). Supplementation with other compounds, including N-acetyl cysteine, vitamin C, and flavonoids, should provide protection against oxidative damage to cytosolic proteins such as cytoskeletal and cardiac contractile proteins (153,156,188,205,214,222,231,263). It was, therefore, speculated that increasing the capacity of the myocardium to quench ROS would decrease the deleterious effects of cardiomyocyte protein and lipid damage and protect the myocardium against infarction.

The first question was addressed using western blot analyses to quantify accumulation of HSP72 and HSP73. To address the second and third questions, myocardial protection against I-R-induced injury was assessed by the following: (1) evaluation of myocardial contractile performance using an *in vivo* I-R model and (2) measurement of myocardial cell death (infarction). Additionally, the efficacy of the proposed interventions for increasing myocardial antioxidant capacity was evaluated using measurements of enzymatic and total antioxidant status as well as markers of protein oxidation and lipid peroxidation. All of the above techniques were performed on myocardial tissue from rodents that received either exercise training alone, antioxidant supplementation alone, exercise training plus an antioxidants, or no treatment (control) followed by one of two *in vivo* myocardial I-R protocols that differed in duration.

CHAPTER 2
REVIEW OF RELATED LITERATURE

Introduction

Myocardial ischemia is a disease process characterized by reduced coronary blood flow. Re-establishment of coronary flow is vital for limiting myocardial damage due to ischemia. Reperfusion, however, brings with it the risk of damage resulting from the toxic effects of reactive oxygen species (ROS) (88-89,99,211-212). The cardiotoxicity of ROS is related to the cellular damage resulting from the instability and subsequent reactivity of ROS (89). One purpose of this review is to characterize I-R injury and the potential role of antioxidant compounds in limiting I-R associated damage in the myocardium.

Induction of molecules called heat shock proteins (HSPs) has been correlated with protection of the myocardium during ischemia-reperfusion (I-R). The preferential synthesis of HSPs in response to a variety of stresses is well characterized (176). The physiological stress induced by exercise training is among those stresses capable of inducing expression of heat shock genes (146). Therefore, in addition to reviewing antioxidants and I-R injury, this review will also summarize the current understanding of expression and biological function of HSPs, including their potential role in exercise-induced cardioprotection during I-R.

Myocardial Ischemia-Reperfusion Injury

As stated previously, myocardial ischemia is characterized by decreased coronary blood flow resulting in an inadequate delivery of oxygen and nutrients to the myocardium, mandating prompt re-establishment of coronary flow to limit ischemic damage. Reperfusion, however, is associated with myocardial injury resulting from the damaging effects of ROS and their participation in degenerative cellular processes including lipid peroxidation, protein oxidation, and DNA damage (88,89,99,211-212). The high prevalence of coronary artery disease demands that research efforts be focused on developing strategies for protecting the myocardium from damage resulting from coronary occlusion and reperfusion. To develop effective strategies, one must first understand the cellular mechanisms involved in myocardial I-R that contribute to impaired myocardial function and infarction.

Two distinctly different types of I-R have been described; myocardial stunning and myocardial infarction. Myocardial stunning can be defined as the mechanical dysfunction resulting from I-R despite the restoration of coronary flow and the absence of irreversible damage. Myocardial stunning generally follows ischemia lasting less than 20 minutes (21). Myocardial infarction, on the other hand, is characterized by irreversible myocardial cell damage or death following persistent or sustained ischemia (21). While these two injuries are distinctly different, it is unknown whether they share mechanisms.

The mechanisms thought to contribute to myocardial cell dysfunction and death during I-R are numerous and not completely understood (7). Among the primary pathogenic agents, however, are ROS (17-20,76-78,163,219,265-266). Other mechanisms that have also been shown to promote myocardial damage include calcium overload due to impaired ATP production (7); increased proteolytic activity associated with disruptions in calcium homeostasis (228-229); and platelet/leukocyte aggregation and activation resulting in further coronary occlusion and release of coronary vasoconstrictors such as thromboxanes (136,229). A brief summary of the role of ROS in I-R induced injury and the potential benefit of antioxidants in providing protection against I-R injury follows.

Reactive Oxygen Species and Ischemia-Reperfusion Injury

Sources of reactive oxygen species

ROS such as the peroxyl radical ($ROO^{\cdot}$), the superoxide anion ($O_2^{\cdot -}$), the hydroxyl radical ($HO^{\cdot}$), and hydrogen peroxide (H_2O_2) are derived from the univalent reduction of molecular oxygen (90). Certain ROS are known as free radicals because they contain one or more unpaired electrons in their outer orbital (e.g. $ROO^{\cdot}$, $O_2^{\cdot -}$, $HO^{\cdot}$). When a ground state O_2 molecule accepts a single electron, the product is $O_2^{\cdot -}$ and addition of a second electron yields H_2O_2. Homolytic fission of the O-O bond in H_2O_2 produces two $HO^{\cdot}$ molecules and can be achieved by a simple mixture of H_2O_2 and an iron (ferrous) salt. Called the Fenton reaction, the $HO^{\cdot}$ produced by this fission is capable of reacting at very high rate constants with almost every cellular component (145).

Potential sources of ROS during I-R are the mitochondrial respiratory chain, xanthine oxidase activity, enzymatic arachadonic acid oxygenation, nitric oxide synthesis, catecholamine oxidation and the neutrophil oxidative burst (110). Another implicated mechanism is the stress associated with oxygen deprivation during ischemia (191). Of these, the enzymatic oxidation by xanthine oxidase of purines such as xanthine and hypoxanthine is believed to be one of the major sources of ROS during I-R in rodents (35,62). Mainly located in the vessel walls of most tissues, including cardiac muscle, the enzyme xanthine dehydrogenase (XDH) catalyzes the oxidation of hypoxanthine to xanthine, and xanthine to uric acid. During I-R, XDH may either reversibly or irreversibly be transformed to xanthine oxidase. In contrast to its dehydrogenase form, xanthine oxidase utilizes O_2 as the electron acceptor and produces $O_2^{\bullet -}$ while catalyzing the oxidation of hypoxanthine to uric acid (100). In the post-ischemic myocardium, activated neutrophils are also known to release large amounts of ROS that have also been associated with I-R injury (219). The relative contribution of any of these sources of ROS in the pathogenesis of myocardial dysfunction remains to be defined.

The cardiotoxicity of ROS is largely reflective of their rapid participation in damaging reaction with cellular components (89). The damage resulting from these reactions include lipid peroxidation, protein oxidation and DNA damage (219). During myocardial I-R, cardiac proteins and cell membranes are particularly vulnerable to oxidative damage (88-89,99,211-212). An overview of oxidative damage to myocardial lipids and proteins follows.

Myocardial lipid peroxidation

Polyunsaturated fatty acids (PUFAs) are highly susceptible to ROS attack and oxidative damage at their sites of unsaturation. Briefly, the attack begins when ROS have sufficient energy to abstract H atoms from the methylene groups of PUFAs forming lipid peroxyl radicals that can then react with other PUFAs, starting an amplifying chain reaction (4). Peroxyl radicals are also capable of propagating further oxidative damage to other membrane-associated molecules such as proteins. The overall result of this attack is destruction of membrane PUFAs leading to alteration of membrane fluidity and permeability. Compromised cell membrane integrity has been called the most destructive reaction that occurs during I-R, possibly contributing to cardiac dysrhythmia and cell death (219,229). By-products of lipid peroxidation such as malondialdehyde, lipid hydroperoxides, conjugated dienes, and isoprostanes are often measured to estimate the extent of ROS-mediated tissue damage (90).

Myocardial protein oxidation

Included in the ROS-mediated damage to cardiac proteins is oxidation of contractile, cytoskeletal, and membrane bound proteins and enzymes (4,99). Quantitative studies of protein oxidation reveal that proteins containing sulfhydryl groups are highly susceptible to damage (4). Much of the damage caused by protein oxidation is irreparable and proteins that have been damaged by ROS are susceptible to further proteolytic cleavage. Oxidation of proteins results in formation of carbonyl groups; assay of protein carbonyl content has been used as an estimation of oxidative damage to proteins (207).

The combined effects of lipid peroxidation and protein oxidation have a profound, detrimental effect on the structure and function of the cardiac myocyte (229). However, cardiomyocytes have intrinsic mechanisms designed to defend against oxidative damage. The following sections will highlight key components of the myocardial antioxidant defense system.

Antioxidant Defense Mechanisms

Cells have extensive endogenous and exogenous mechanisms for providing protection against damage by reactive oxygen species. Among the cells' primary defenses are the enzymatic antioxidants which include (1) superoxide dismutase, which dismutates the superoxide radical; (2) catalase, which decomposes hydrogen peroxide; and (3) glutathione peroxidase, which decomposes hydrogen peroxide with the assistance of the nonenzymatic antioxidant glutathione (90,263). Other non-enzymatic antioxidants include vitamin E, vitamin C, carotenoids, glutathione, ubiquinones, and flavonoids. Antioxidants work both independently and synergistically to maintain a reduced cellular environment (199,263).

Given the potential role of ROS in I-R injury, interventions designed to increase the cells' antioxidant defenses are a promising strategy for minimizing I-R associated cellular injury (8,69,72,74,86-87,124,128,158,166,201,218). While increases in cellular enzymatic antioxidants can be achieved following stresses including exercise training (197,199), another effective method for increasing antioxidant defenses is via dietary supplementation with nonenzymatic antioxidants. The primary nonenzymatic antioxidants pertinent to this study and their roles in defending against radical-mediated damage are reviewed in the next section.

Vitamin E

The term vitamin E refers to at least eight structural isomers of tocopherol (15). Of these isomers, α-tocopherol is believed to possess the most potent antioxidant activity (31). Protection of membranes by vitamin E is dependent upon the incorporation of α-tocopherol into cellular membranes (233).

Vitamin E is the most widely distributed antioxidant in cells and is of particular importance because of its ability to convert peroxyl radicals to less reactive molecules. Vitamin E can also break the lipid peroxidation chain reaction that occurs during free radical damage and is, therefore, considered the primary chain-breaking antioxidant in cell membranes (31,261). While vitamin E is an efficient radical scavenger, the interaction of vitamin E with a radical results in a reduction of functional vitamin E and the formation of a vitamin E radical. It is postulated that the capacity of vitamin E to serve as an antioxidant is dependent upon other antioxidants that are capable of recycling vitamin E during periods of oxidative stress. Antioxidants such as vitamin C and glutathione, reviewed later in this section, are among those capable of recycling the vitamin E radical (29,116-118,180).

The concentration of vitamin E in cell membranes is relatively low under most conditions (110). Hence, numerous investigations have attempted to elevate tissue concentrations. At high doses however, the intestinal absorption of dietary vitamin E decreases (152). As the oral dose of radioactive alpha tocopherol increases, the percent absorption decreases from 70% at RDA levels (75 IU/kg diet) to 30% at very high dose of 20,000 IU/kg diet (51). Rats supplemented with 10,000 IU vitamin E/kg diet for up to

20 weeks have significantly elevated tissue and serum tocopherol concentrations with no signs of toxicity (43-44,119,154). Concentrations of vitamin E in the heart did, however, appear to peak at approximately 14 weeks of supplementation (43-44,154).

Because vitamin E possesses many of the criteria considered to be a good antioxidant, numerous investigations have examined the effects of vitamin E on I-R injury in the heart. Whole organ and animal models of myocardial ischemia, as well as ischemic heart disease patients, have been studied to investigate whether vitamin E might be effective in preventing post-ischemic myocardial dysfunction/necrosis. Vitamin E treatment of *in vitro* hypoxic rat hearts has been shown to be effective in preventing irreversible cardiomyocyte injury with reoxygenation, as well as reducing the contractility and mitochondrial derangements (69,163,194,199). Using isolated-heart models, vitamin E has been shown to quench lipid radical formation during reperfusion (8,72). A 51% increase in the myocardial content of vitamin E *in vivo* protects rat hearts against *in vitro* I-R induced injury (158, 240). The results of these isolated heart studies constitute compelling evidence that cardiac vitamin E protects against myocardial I-R injury *ex-vivo.*

Only recently have efforts been focused on the cardioprotective effect of vitamin E using *in vivo* models of I-R. Infusion of vitamin E prior to *in vivo* I-R was reported to limit myocardial infarction in pigs, whereas the same dose of vitamin E administered only during ischemia was less effective (124). Vitamin E has also been shown to decrease the incidence of ventricular fibrillation during chronic ischemia in rat models (74,220). These studies suggest that vitamin E is effective as a cardioprotectant against the irreversible injury associated with I-R.

Vitamin C

Vitamin C (ascorbic acid or ascorbate) is a naturally occurring antioxidant, found abundantly in many plants consumed regularly by humans. In contrast to vitamin E, vitamin C is a hydrophilic antioxidant that exerts its radical scavenging activities in the aqueous compartments of cells (263). Vitamin C scavenges superoxide, peroxyl, and hydroxyl radicals directly, and also plays an important role in recycling the vitamin E radical back to its active form (36,188,231,263). Like vitamin E, in the process of scavenging radicals the radical form of vitamin C is generated and must be recycled to its reduced form (i.e., via glutathione) to continue exerting its antioxidant activities. The ability of vitamin C to exert pro-oxidant effects is of concern when concentrations of the vitamin are relatively high (i.e., greater than 1 mM). This pro-oxidant activity arises from the reaction of vitamin C with transition metals such as $Fe+^3$, resulting in reduction of $Fe+^3$ to the $Fe+^2$ state, which can, in turn, induce radical generation by reactions previously reviewed (246,258). Hence, careful consideration must be given to vitamin C dosage when supplementing for the purpose of preventing oxidative damage (263).

The protective effects of supplemental vitamin C, as well as synergistic effects of vitamins C and E, have been investigated extensively. Supplementation with vitamin C has been shown to increase the global antioxidant capacity of heart tissue and protect a variety of tissues and cells against oxidative injury (5,155,210). Supplementation with both vitamins C and E appears to lead to a sparing effect on vitamin E as well as protection against oxidative injury (36,188,231). Accordingly, vitamin C deficiency induces a greater susceptibility to oxidative injury, perhaps to a greater extent than does vitamin E deficiency (231). Further, supplementation with a combination of vitamins C and E may help prevent the potential pro-oxidant effects of larger doses of vitamin C (36).

Glutathione

Glutathione (GSH), a tripeptide synthesized primarily in the liver from glutamate, cysteine, and glycine, is the most abundant nonprotein thiol in cells (168). GSH is a versatile molecule, serving multiple roles in defending cells against ROS-mediated damage. Perhaps its most important role in antioxidant defense is serving as a substrate for glutathione peroxidase (GPX) which catalyses the enzymatic reduction of hydrogen peroxide and other organic peroxides to water and alcohol, respectively. In serving as a substrate for this antioxidant enzyme, two molecules of GSH are oxidized to form the disulfide form of glutathione (GSSG). GSSG is then recycled back to the reduced form by the enzyme glutathione reductase with NADPH providing the reducing power (111,113). GSH can also serve as a direct scavenger of hydroxyl, superoxide, and carbon based radicals, as well as singlet oxygen (263). Finally, GSH serves an important role in recycling the radical forms of other antioxidants (i.e., vitamins E and C) to restore their radical-quenching capacity (187).

Given the many functions of GSH in maintaining a reduced cellular environment, it is not surprising that alterations in GSH content can alter cell function. Using L-buthionine-[S,R]-sulfoximine (BSO), an inhibitor of the enzyme γ-glutamyl synthase, myocardial GSH concentrations can be significantly decreased (132,168). Starvation-induced and BSO-induced decreases in GSH are associated with accelerated production of malondialdehyde, a product of lipid peroxidation (132). Importantly, GSH deficiency has been reported to alter antioxidant enzymatic activity (135) and to impair recovery of contractile performance following myocardial ischemia (251). In contrast, N-acetyl cysteine (NAC; a source of cysteine for GSH synthesis) administered intraperitoneally, has been shown to result in increases in tissue GSH concentration and improvements in muscle endurance (205,222). However administration of GSH and GSH ethyl esters don't appear to increase GSH concentrations or to alter redox status (133).

Carotenoids

Carotenoids, or carotenes, are colored pigments found in yellow and green vegetables. Some carotenoids, such as β-carotene, are precursors to vitamin A, though no RDA has been established for them. Many members of the carotenoid family are chain-terminating antioxidants and can quench singlet oxygen in addition to a variety of other radical species; β-carotene, lycopene, astaxanthin, and canthaxanthin are among those species with well-documented antioxidant activity (30,213). Which carotenoid possesses the greatest radical scavenging activity remains an issue of some controversy. Historically, it was thought that β-carotene was the most effective antioxidant among the carotenes. However, some investigations have suggested that carotenoids with oxo groups at the 4 and 4' positions in the beta-ionine ring (i.e., astaxanthin, and canthaxanthin) are more effective radical scavengers than β-carotene (213). In contrast, other investigations have found β-carotene and lycopene to have greater scavenging activity than astaxanthin, and canthaxanthin (174). Assessment of carotenoid activity *in vivo* is limited by the lack of good models. Use of varying *in vitro* radical generating systems and methods for measuring scavenging activity undoubtedly account, at least in part, for these contradictory reports.

Importantly, carotenoids may act synergistically with other antioxidants. For example, several reports exist demonstrating that as oxygen tension varies, β-carotene and α-tocopherol may have varying importance as chain-breaking antioxidants (28,213). This issue remains somewhat debated with other reports of similar protection against peroxidation in tissue homogenates and microsomal membrane preparations with β-carotene and α-tocopherol supplementation (189,253-254). Whether β-carotene can recycle the vitamin E radical continues to be debated (162,213). Similar vitamin E, carotenoids with two polar groups distally situated on the molecule (i.e., β-carotene, astaxanthin, and canthaxanthin) can span phospholipid membranes and provide membrane stability (213).

Unlike retinoids, which have a well-described associated toxicity, β-carotene is relatively nontoxic even when plasma concentrations are elevated nearly 100-fold above normal levels. The only observed side effect in clinical trials has been hypercarotenodermia, a condition of yellowing of the skin (10,60). Chronic administration of enormous doses to dogs and mice has resulted in reported nonspecific histological changes in liver (103). Canthaxanthin supplementation, however, has been associated with reports of a reversible retinopathy (96).

Flavonoids

Flavonoids are a large family of diphenylpropanes (over 4000 members identified) that are commonly found in plants consumed by humans. Family members include, but are not limited to, flavones, isoflavones, flavanones, anthocyanins, and catechin (49). Flavonoids have been reported to possess a wide variety of biological activities ranging from inhibition of inflammatory enzymes (lipoxygenase, cyclooxygenase, xanthine oxidase, NADH-oxidase, phospholipase A_2, protein kinase) to antitumoral, antiviral, antimutagen, antiinflammatory, antiischemic, and antiallergic activities (32). Many of these biological effects are thought to be a result of the antioxidant capacity of flavonoids (23,156,214). Radical scavenging activities of flavonoids appear to vary greatly among family members, but include quenching of peroxyl, hydroxyl, and superoxide radicals as well as hydrogen peroxide and a variety of chemically generated radicals not naturally found in the body (32).

Among the many flavonoids under investigation for their potential role in protecting against radical-mediated disease processes is the polyphenolic flavonoid family member, catechin, which is found in significant concentrations in green and black tea and red wine. Catechin has been shown to efficiently scavenge superoxide radicals, inhibit metal ion-mediated radical formation, and to inhibit formation of lipid peroxyl radical species (215,223). In addition to inhibiting lipid peroxidation, catechin was reported to prevent the radical-mediated depletion of vitamin E and β-carotene in human plasma in a dose-dependent manner (153).

Ubiquinones

Ubiquinones are naturally occurring lipid-soluble derivatives containing an isoprene or farnesyl tail which, in their reduced forms, are highly efficient antioxidants (66,169). Ubiquinone-10, often called coenzyme Q_{10}, is the primary isoform in most mammalian species (66). It is found primarily in the reduced form, with approximately 50% of total cellular stores found in mitochondrial membranes (3). Here, ubiquinones serve a well-know role as a redox component of the mitochondrial electron transport system, in which they act as "couplers" between NADH dehydrogenase or succinate dehydrogenase and cytochrome b in the respiratory chain (227).

In addition to the role of coenzyme Q_{10} in mitochondrial electron transport, this phenol-ring containing structure can also scavenge oxygen radicals and singlet oxygen, thereby protecting membrane lipids from peroxidation (66). Reduced ubiquinones can also exert antioxidant effects indirectly by regenerating vitamin E from its radical form via an NADPH-dependent cycle (66,237).

The protective role of coenzyme Q_{10} was first described in 1966 when lipid peroxidation was inhibited by coenzyme Q_{10} in isolated mitochondrial membranes (169). Exogenous coenzyme Q_{10} has been reported to be incorporated into cellular and subcellular membranes such as the sarcoplasmic reticulum and the mitochondrial membranes (169). Further, coenzyme Q_{10} has been reported to protect heart and liver tissue against lipid peroxidation induced by exposure to oxidants (223). Oxidant-induced decreases in GSH and vitamin E were prevented by coenzyme Q_{10} in ischemic tissues (120).

Despite reports of limited tissue uptake of orally supplemented coenzyme Q_{10} in rats (264) the role of coenzyme Q_{10} in protecting the myocardium from I-R induced injury has also been investigated using isolated perfused rat heart models. Following coenzyme Q_{10} supplementation, left ventricular developed pressure was improved with increases in coenzyme Q_{10} concentration found only in the plasma, not heart tissues (151). In contrast, in a similar study using an isolated perfused heart model in pigs and rats, coenzyme Q_{10} supplementation was associated with significant increases in tissue and mitochondrial coenzyme Q_{10} concentration, with a concomitant decrease in lipid peroxidation by-products and improvement in post-ischemic ventricular recovery (68,161,262). Thus, the protective role of coenzyme Q_{10} in cardioprotection during I-R remains equivocal and may be dependent upon species, dose, and/or duration of supplementation.

Heat Shock Proteins: An Overview

One of the best described cellular responses to stress, is the induction of highly conserved stress proteins called heat shock proteins (HSPs) (63,142-143,177,209,238). The heat shock response, defined as the rapid induction of HSPs, was first identified in *Drosophila* nearly 40 years ago (209). This initial observation led to the characterization of a rapidly increasing number of isoforms as members of HSP families.

Induction of HSPs occurs not only in response to heat stress, but also in response to a variety of environmental and physiological stresses including hypoxia, altered pH homeostasis, exposure to oxidants and transition metals, ischemia/reperfusion, and presence of amino acid analogs (63,104,139,143,170,172,247,248-250). Thus, this generalized cellular response to environmental and physiological disturbances is also referred to as the "stress response" rather than the more limited term, "heat shock response" (63). Importantly, recent studies have revealed that the physiological stress associated with endurance exercise training also results in rapid induction of some isoforms of HSPs in rodents (53,55,129,146,149-150,152,198,216,235). Exercise induction of HSPs will be discussed in detail later in this review.

Overview of HSP families

Based on their migration during gel electrophoresis, five main groups of HSPs have been characterized: (1) small HSPs, 8 to 32 kDa; (2) 50-60 kDa HSPs; (3) 70 kDa HSPs; (4) 90 kDa HSPs; and (5) 100kDa plus HSPs (126). HSP isoforms vary between organisms and even between cell types within an organism; however, all organisms express 65-78 kDa HSPs that belong to the HSP70 family (142). Since the focus of the proposed experiments is on HSP72, only a brief overview of other families of HSPs is provided in this review.

Five prominent members of the small HSPs family include ubiquitin, ∝B-crystallin, HSP27, HSP20, and HSP32. Ubiquitin is approximately 8-kDa, highly conserved, and important in both chromatin structure and in protein degradation. (33,249). ∝B-crystallin, a 22-kDa protein, belongs to a family of crystallins first identified in vertebrate lens tissue but also found in cells with high mitochondrial content such as cardiac and type I and IIa skeletal muscle fibers (181). ∝B-crystallin functions as a molecular chaperone, facilitates protein folding, and prevents aggregation of denatured proteins. Constitutive expression of HSP27 appears to parallel growth and be under cell cycle regulation (249). Similar to αB-crystallin, HSP27 is found in relatively high concentrations in heart tissues and appears to have a role in regulation of muscle cell contraction (50,221). HSP20 has been isolated from rat and human skeletal muscle and shows sequence homology with both HSP27, and αB-crystallin. Limited information about the biological function of HSP20 exists. HSP32 is an inducible form of heme oxygenase, referred to as heme oxygenase 1 (HO-1), and is induced readily by oxidizing agents including reactive oxygen species. Both HO-1 and the constitutive isozyme HO-2, generate the cellular messenger carbon monoxide (121-122,160,241).

The 50-60 kDa HSPs family contains two distinct subgroups; the GroE subgroup found in the eubacteria, plastids, and mitochondria, and the TCP-1 subgroup found in the eukaryotic cytosol and the archebacteria. Both chaperone subfamilies assist the correct folding of nascent polypeptides in the cytosol (63-65,81)

HSP90 is the most abundant constitutively expressed stress protein in eukaryotic cytosol. HSP90 is bound to a wide range of proteins such as viral tyrosine kinases, steroid hormone receptors, and other heat shock factors (200). In addition to regulation of the proteins it binds, HSP90 is thought to play a role in assisting the folding of nascent polypeptides (247).

Both HSP100 and HSP110 are normal constituents of mammalian cells and are generally expressed at low levels (225). Though both proteins are inducible, their biological functions remain poorly defined.

The 70 kDa-HSPs, the family of proteins most important to the proposed experiments, are highly conserved, abundant proteins estimated to account one to two percent of all cellular protein (101). HSP70 genes, first visualized as chromosomal puffs in *Drosophilia*, encode a multigene family of proteins in eukaryotes (209). At least four groups of genes make up the HSP70 family, the most prominent members of which are HSP72 and HSP73, located in both cytoplasmic and nuclear regions of the cell. HSP73, constitutively expressed in all mammalian cells, is also commonly referred to as the

HSP70 cognate protein, or HSC73. Conversely, HSP72 synthesis is primarily restricted to cells experiencing stress and is, hence, called the inducible form of HSP70. While the synthesis of these two 70kDa HSPs is distinctly different, they exhibit high sequence homology (~95%) and similar biochemical properties.

Two other major members of the HSP70 family are the mitochondrial, and sarcoplasmic glucose-regulated proteins (GRP), GRP75 and GRP78, respectively (179,249). GRPs received their name upon observation that they are induced by glucose starvation.

Biochemical roles of 70-kDa HSPs

The complex physiological roles of the HSP70 family of proteins are only beginning to be unraveled. Numerous studies have shown that HSP72 and HSP73 interact with variety of proteins to serve a role in protein maturation, protein transport, and proteolysis, as well as in protection against a variety of stresses via unknown mechanisms (83,146,249). Some of the biochemical functions of 70 kDa HSPs are briefly reviewed below.

Proteins are synthesized on ribosomes, targeted to specific subcellular compartments, folded and modified to assume an active conformation, and, eventually degraded. HSPs, particularly HSP72, play a vital role in this protein lifecycle. Optimal functioning and maintenance of any cell is explicitly dependent upon the presence of correctly folded proteins; therefore, chaperoning function of HSPs and particularly HSP72 is very important (97). To carry out its role as a “molecular chaperone” HSP72 associates with nascent polypeptide chains on ribosomes and orchestrates the stabilization and folding of these new proteins (9,56,73). Post-translational protein modification, sorting, and translocation into targeted cellular compartments (i.e. endoplasmic reticulum or mitochondria) also requires the action of HSP70 family members (25,200,206). HSP70 proteins are also involved in proteolysis via recognition of specific amino acid sequences in damaged proteins (42,57). By recognizing damaged proteins, HSPs may then target them for lysosomal degradation or prevent protein aggregation thus facilitating accessibility to other proteolytic systems (42,57,173).

Regulation of HSP induction

Transcription of HSP genes is mediated by heat shock transcription factors (HSF) (192,256). In unstressed cells, HSF exists as a monomer, incapable of binding DNA. In response cellular stresses, HSF oligomerizes and becomes active (217,256). Active HSF can then bind specific elements within HSP gene promoters referred to as the heat shock element (HSE) (257). HSE binding results in transcription of genes encoding HSPs (67,175-176,193,255-256). The activation of the DNA binding form of HSF can be detected within minutes of temperature elevation (178). HSP72, shown to interact with HSF1 and has, thus, been implicated in the regulation of its own transcription factor (1). That is, under unstressed conditions, HSP72 may interact with HSF to maintain it in a non-DNA binding (monomeric) state. During cellular stress, the appearance of denatured proteins creates a pool of newly synthesized proteins that compete with HSF for association with HSP72. Therefore, the appearance of denatured proteins results in the release of HSF from HSP72; the released HSF oligomerizes and then binds to DNA. The activation of HSF-DNA binding leads to an elevation of transcription and synthesis of HSP72 (176,178).

HSP72 and cytoprotection

While the early evidence that HSP72 was cytoprotective was correlative in nature, more recent studies, using both transfection of the HSP72 gene into cultured cells and transgenic mouse models, have provided direct evidence that HSP72 provides cellular protection against a wide range of stresses (6,11,26,79,107,123,140-141,144,157, 171,195). For example, transfection of cells with a plasmid construct consisting of the human HSP72 gene resulted in increased basal concentrations of HSP72 and thermotolerance (6,82). Similar results have been reported with transfection studies using rat fibroblasts and myocytes (98,141). Further support of the notion that HSP72 is cytoprotective came from studies in which cells injected with HSP72 antibodies to block the heat stress-induced nuclear translocation of HSP72 rapidly died following exposure to a subsequent hyperthermic stress (81,208).

In addition to cultured cells, it well established that heat shock proteins are readily synthesized in cardiomyocytes *in vivo* (75,109). In an early study, the core body temperature of rodents was elevated to 42°C-42.5°C for 15 minutes followed by infusion of [35S]-methionine during the 2 hour recovery (46). Measurement of labeled proteins revealed that there was a depression in overall protein synthesis despite an increase in the synthesis of HSP72 (46,92).

Ischemia and reperfusion have also been shown to induce HSP72 in cardiomyocytes (167,170). Six hours of left anterior descending artery occlusion in the dog heart resulted in increased HSP72 expression (59). Not long after this observation was the realization that HSP72 mRNA increases after as few as 5 minutes of myocardial ischemia in rabbit hearts (125). Repetitive short duration ischemia and reperfusion, often called "ischemic preconditioning" (four sets of 5min ischemia and 5 min reperfusion) increases myocardial HSP72 in as soon as 2 hours, with maximal accumulation observed 24 hours after the last reperfusion period (50,157,231).

HSP72 and protection against myocardial ischemia-reperfusion injury

Compelling evidence implicates HSP72 in cardioprotection during I-R. Numerous investigations have demonstrated that heat stress-induced myocardial HSP72 confers enhanced post-ischemic contractile function and decreased myocardial infarction size following coronary occlusion and reperfusion *in vivo* (26,47,53-54,58,61,106,157) as well as an isolated perfused heart model (45,243). In this laboratory, pre-treatment with heat stress resulted in improved maintenance of myocardial contractility during *in vivo* I-R with an associated 500% increase in myocardial (53). While it is clear that a whole-body heat stress can result in a variety of cellular changes in addition to induction of HSP72, the amount of HSP72 present following whole-body heat shock has been directly related to the degree of myocardial protection obtained (107).

Perhaps the most compelling evidence that HSP72 provides cardioprotection during I-R are the experiments using transgenic mice overexpressing HSP72. Hearts from these transgenic mice have demonstrated improved post-ischemic contractile function, decreased infarction size, and attenuated creatinine release following I-R (26,106,157,195). Similarly, overexpression of human HSP72 in rat myogenic cell lines via transfection confers protection against metabolic stress (252) and ischemia (171).

Exercise-induced HSP72 expression and myocardial protection

A number of myocardial benefits have been attributed to regular physical exercise, yet the exact mechanism(s) for exercise-induced protection to the myocardium continues to be debated (22). Interestingly, both chronic (i.e., 10-12 weeks) and acute (1-5 days) treadmill exercise can induce HSP72 in a variety of rodent tissues including the myocardium (53,148-150,198,224). Surprisingly, as little as one bout of exhaustive running in rats has been shown to increase HSP72 mRNA and protein in both heart and skeletal muscle (216). Hence, while the mechanisms behind exercise-associated cardioprotection remain largely unknown, compelling evidence exists that attributes at least part of the myocardial protection associated with exercise to increases in expression of HSP72 (53,148-150,198).

The multiple physiological changes imposed by exercise are well-described (22,146). However, the signal (s) by which HSP72 is induced during exercise remains unclear. Possible mechanistic links between exercise stress and HSP72 induction include (1) elevation of tissue temperature; (2) acceleration of ROS production; (3) disruption of intracellular pH; (4) perturbation of calcium homeostasis; (5) progressive depletion of glucose and glycogen stores; or (6) a combination of two or more of these biochemical alterations (146,148,216).

Evidence of the relationship between exercise-associated cardioprotection and HSP72 expression is growing (52-54,146,148,198). In a study by Locke et al., hearts from control, heat stressed, and exercised (3 consecutive days of treadmill running) rats were subjected to global I-R. The results revealed that an improved functional recovery in both the exercised and heat stressed animals was associated with large increases in myocardial HSP72 (149).

Our laboratory has completed a series of experiments to elucidate the effects of both acute and chronic exercise on cardioprotection during *in vivo* I-R. Chronic endurance training (10-12 weeks of treadmill running) resulted in a 500% increase in rat myocardial HSP72 (54,198). Consistent with previous experiments employing *in vitro* models, HSP72 induction was associated with improved contractile performance during ischemia and reperfusion as well as protection against myocardial lipid peroxidation (54,198). Acute exercise training (3-5 consecutive days of treadmill running) yielded a similar outcome, with significant induction of myocardial HSP72 paralleled by improved left ventricular developed pressure and rate of pressure development during *in vivo* I-R (54). Interestingly, however, short-term exercise training in a cold environment abolished the accumulation on HSP72 associated with exercise, but did not attenuate the cardioprotection during *in vivo* I-R (91).

Summary

While I-R injury to the myocardium is a significant clinical problem, the potential for effective interventions is promising. Among the possible protective strategies are those designed to improve antioxidant capacity and those that induce heat shock proteins. Recent evidence suggests that exercise-induced increases in HSP72 are associated with cardioprotection during I-R. Further, enhanced antioxidant defenses via dietary supplementation can provide protection against the ROS-mediated injury associated with I-R. However, the combined effects of exercise training and antioxidant supplementation on myocardial I-R injury are unknown. An increased production of ROS has been implicated as an exercise-induced signal for up-regulation of HSP72. Hence, while each intervention, independently, can protect the heart against I-R injury, it is possible that combining dietary antioxidant supplementation with exercise training could have an antagonistic effect (84,239). Therefore, investigation of the individual and combined effects of short-term exercise training and supplementation with nutritional antioxidants on the expression of HSP72 and protection against myocardial ischemia-reperfusion injury is warranted.

CHAPTER 3
METHODS AND PROCEDURES

Animals and Experimental Design

Female Sprague-Dawley rats (3 months old) were divided into two dietary groups: (1) control and (2) antioxidant supplemented. Animals in the control group were fed a diet designed to meet the National Research Council's recommended requirements for macro- and micronutrients (AIN93M) in the adult rat (203). The animals in the antioxidant-supplemented group were fed the AIN93M diet supplemented with the 7 antioxidants listed in Table 1. The diets were professionally prepared by Bio-Serv (Frenchtown, NJ).

Table 1. Antioxidant diet composition

Antioxidant added to AIN93M rodent diet	**per kg of rodent diet**
vitamin E	1000 IU
selenium	0.3 mg
ascorbic acid	1000 mg
20% beta-carotene	225 mg
coenzyme Q_{10}	30 mg
N-acetyl cysteine	200 mg
catechin	100 mg

After completing a 6-week feeding protocol, rats within each dietary group were further randomized to receive five consecutive days or treadmill training (following one week of habituation to the treadmill) or to remain sedentary. Finally, following a total of eight weeks of feeding, each of these groups was further randomized into one of three surgical treatment groups: (1) sham surgery; (2) short-term *in vivo* I-R (20 minutes ischemia/30 minutes reperfusion); or (3) long term *in vivo* I-R (60 minutes ischemia/120 minutes reperfusion). Sham surgery animals, which were used for assessment of antioxidant capacity, underwent all surgical procedures with the exception of coronary artery occlusion and reperfusion. The 20-minute ischemia/30-minute reperfusion group provided a model of myocardial stunning while the 60-minute ischemia/120-minute reperfusion group, in contrast, served as a model for myocardial infarction (figure 1).

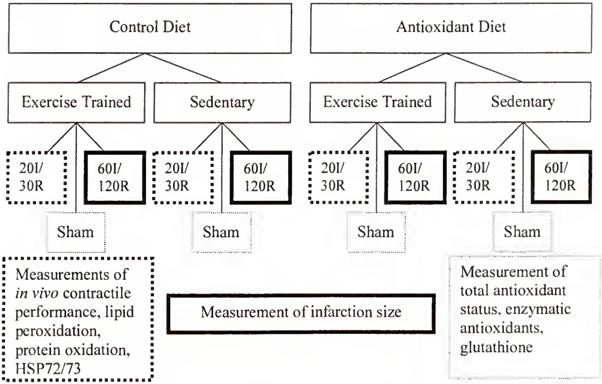
Control Diet
Antioxidant Diet
Exercise Trained
Sedentary
Exercise Trained
Sedentary
20I/ 30R
60I/ 120R
20I/ 30R
60I/ 120R
20I/ 30R
60I/ 120R
20I/ 30R
60I/ 120R
Sham
Sham
Sham
Sham
Measurements of *in vivo* contractile performance, lipid peroxidation, protein oxidation, HSP72/73
Measurement of infarction size
Measurement of total antioxidant status, enzymatic antioxidants, glutathione

Figure 1. Experimental Design; 20I/30R=20 min ischemia/30 min reperfusion; 60I/120R = 60 min ischemia/120 min reperfusion; Methods to be used for analysis of the eight surgical groups are indicated by dotted, dashed, or solid bold boxes.

Exercise Training Protocol

The animals randomized to exercise training groups performed a short-term training protocol beginning with five days of habituation to the motorized treadmill, each with increasing exercise duration (i.e., 10 minutes on day one, 20 minutes on day two, etc., until 50 minutes is achieved on day five). Following this habituation period, animals underwent five consecutive days of treadmill running (60 minutes/day) at 30 meters/minute or approximately at 60-70% of $VO_{2\,max}$ (131,227). This protocol has been shown to induce myocardial HSP72 expression in adult rats (53,55,197-198).

In Vivo Myocardial Ischemia-Reperfusion Protocol

At the completion of the feeding and exercise protocols (24 hours following the final exercise bout in trained animals), rats were anesthetized with 40 $mg \cdot kg^{-1}$ sodium pentobarbital, intubated with a 14-gauge polystyrene tube by tracheostomy, and ventilated with room air using a small animal ventilator (Columbus Instruments, Columbus,Ohio). Rectal temperature was monitored via a thermistor probe throughout the experiments (Yellow Springs Instruments, Yellow Springs, Ohio). Body temperature was maintained at approximately 37°C with a heated operating platform. Both arterial blood gas and pH homeostasis were carefully maintained throughout the long-term surgical protocol. The surgeon performing the I-R protocol was blinded to the experimental groups.

The heart was accessed via left thoracotomy and a ligature was placed around the left anterior descending coronary artery, close to its origin. Coronary occlusion was achieved by passing both ends of the ligature through a small plastic tube that was then pressed on the surface of the heart directly above the left anterior descending coronary artery. Coronary occlusion was considered successful with the appearance of frank cyanosis on a major portion of the left ventricular wall. The resulting arterial occlusion was maintained for either 20 or 60 minutes by clamping the plastic tube and ligature with small hemostats. Reperfusion was achieved by removing the clamp and the tube and maintained for either 30 minutes or 120 minutes. Reperfusion was considered complete with the observation of immediate resolution of cyanosis. This *in vivo* I-R technique has been used successfully in this laboratory for five years (198).

Measurement of Cardiac Contractile Function

Measurements of cardiac function *in vivo* were performed prior to ischemia and throughout ischemia and reperfusion. Cardiac function measurements included left ventricular developed pressure (LVDP), rate of ventricular pressure development and relaxation (±dP/dt), and electrocardiographic function. Ventricular pressures were measured by placing a catheter filled with heparinized saline into the left ventricular cavity via the carotid artery using techniques described by Geenen et al. (80). The catheter was attached to a pressure transducer interfaced with a computerized heart performance analyzer (Digi-Med, Louisville, KY). A standard limb lead ECG (lead II) was monitored continuously using an amplifier (Grass Instruments, Quincy, MA) and an oscilloscope (BK Precision, Placentia, CA). Rats with more than 3 episodes of ventricular fibrillation, or an episode of ventricular fibrillation that persists longer than 30 seconds, were eliminated from the study. In rats exhibiting ventricular fibrillation, cardioversion was attempted first by gentle massage of a non-cyanotic region of the heart and then by electrical pacing (3-5 v/0.1 ms duration/300 bpm) as necessary.

Assessment of Myocardial Infarction Size

Following completion of the 60 minutes ischemia/120 minutes reperfusion protocol, hearts were prepared for quantification of the area of infarcted tissue using a gross histochemical staining technique (i.e. triphenyl tetrazolium chloride (TTC) tissue enzyme staining technique). Tetrazolium dyes form brick red colored precipitates in the presence of intact dehydrogenase enzyme systems. Infarcted tissue lacks dehydrogenase enzyme activities and is, therefore, left unstained. The reliability and validity of the TTC technique are now well documented (16,70,107).

Briefly, at the end of 120 minutes reperfusion, the coronary artery was re-occluded and 1 ml of Evans blue dye was injected directly into the left ventricle via the left ventricular catheter. After injection of the dye, the heart was removed within 10 seconds, rinsed to remove excess dye, and sliced into ~1.5mm thick cross sections (five slices distal and perpendicular to the occlusion site). Slices were photographed and the areas not stained blue by the Evans dye were interpreted as the "area at risk" for infarction (48). Heart slices were then incubated (within five minutes of removal from the animal) in 1% TTC at 37°C for 20 minutes. Following TTC staining, tissue slices were placed in 10% formalin for ~24 hours. Finally, the formalin-fixed heart sections were photographed and the areas at risk for infarction and infarcted regions were quantified using computerized image analysis in a blinded fashion. Infarct size in each histologic section was expressed as a percentage of the area at risk in each slice.

Tissue Removal, Storage, and Preparation

Tissue removal

At the conclusion of the 20 minutes ischemia/30 minutes reperfusion and the sham surgical protocols, hearts were removed and rapidly frozen for subsequent biochemical analysis. Prior to freezing, the heart was rinsed free of blood using a cold antioxidant buffer containing 50 mM $NaHPO_4$, 0.1 mM butylated hydroxytoluene, and 0.1 mM diethylenetriaminepentaacetic acid (pH 7.4) and sectioned into small portions for ease of analysis without the risk of freeze-thaw damage. Briefly, hearts from the animals undergoing 20 minutes ischemia/30 minutes reperfusion were divided into left ventricular free wall, right ventricular free wall, intraventricular septum, and atrial portions. The left ventricle was further divided into three equal portions via transverse sectioning distal to the occlusion site and roughly perpendicular to the axis of the heart. Sham surgery hearts were freeze clamped using liquid nitrogen and aluminum tongs for optimal measurement of glutathione concentration.

Preparation of protein extract

The distal portions (apex) of the left ventricles from 20 minutes ischemia/30 minutes reperfusion animals were thawed and homogenized in 5 volumes of extraction buffer (10 mM NaCl, 10mM Trizma Base, 0.1mM EDTA, 15mM β-mercaptoethanol, 0.1mM PMSF, 1μM leupeptin, 0.3μM aprotinin, pH 7.6), at 4°C at 5000 rpm using a glass-on-glass homogenizer. Tissue homogenates were centrifuged at 15,000xg for 20 minutes. After determining the protein concentration of the supernatant using the Bradford technique (25), the supernatant was then stored at –80°C for subsequent HSP72 and HSP73 western blotting.

Polyacrylamide Gel Electrophoresis and Western Blotting

Protein extracts from rat heart homogenates was mixed with Laemmli sample buffer to a final of concentration 2.5mg/ml. This mixture was heat denatured for 4 minutes and separated on a 12% polyacrylamide gel (60µg protein per lane) along with a low range pre-stained SDS-PAGE standard. Separated proteins were then transferred to nitrocellulose membranes (0.45µm thick) using the Bio-Rad (Hercules, CA) transfer cell. Membranes were incubated with 3% BSA in TTBS for 2 hours, and then with HSP72 and HSP73 specific monoclonal antibodies for 4 hours (StressGen, Victoria, Canada). The membranes were then incubated secondary antibodies conjugated to horseradish peroxidase for enhanced chemiluminescent detection (Amersham Pharmacia Biotech, Piscataway, NJ). Quantification of the bands from the immunoblots was performed using computerized densitometry. Average intensities of HSP72 and HSP73 bands from control samples were designated as 100% and intensities of individual protein bands were expressed as a percentage of control.

Assessment of Myocardial Antioxidant Status

Biochemical assessment of antioxidant enzyme activity

To determine if the dietary treatments altered endogenous antioxidant enzyme activity, a small sample of the left ventricle from all animals was assayed to determine the activities of total superoxide dismutase (SOD), manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (Cu-ZnSOD), glutathione peroxidase (GPX), and catalase (CAT). Homogenate was prepared in cold 100 mM phosphate buffer w/ 0.05% bovine serum albumin (1:20 wt/vol; pH = 7.4) and centrifuged at 400 x g for 10 minutes. The supernatant was then assayed for total SOD, MnSOD and Cu-ZnSOD using the cytochrome c reduction technique of McCord and Fridovich (164). Selenium GPX activity was then assayed as described by Flohe and Gunzler (71). CAT was assayed using the protocol described by Aebi (2) and modified by Ji et al. (112). Coefficients of variation for SOD, GPX, and CAT assays in this laboratory are ~4, 5 and 3 percent, respectively. These and other spectrophotometric biochemical analyses were performed in triplicate or quadruplicate at 25°C, and samples from all experimental animals were assayed on the same day to avoid interassay variation.

Total antioxidant capacity assay

The assay kit for measuring the total antioxidant capacity was purchased from Randox Laboratory (Antrim, UK). Briefly, a peroxidase (metmyoglobin) reacts with hydrogen peroxide to form ferrylmyoglobin, a free radical species. A chromagen, 2,2'-amino-di-[3-ethylbenzithiazole sulphonate], is incubated with ferrylmyoglobin to produce a radical cation. This cation product has a relatively stable blue green color, which is measured spectrophotometrically at 600 nm using a plate reader (Spectramax Plus, Molecular Devices, Sunnyvale, CA). Antioxidants in the added tissue sample cause suppression of this color production proportional to the antioxidant concentration. The assay is standardized using 6-hydoxy-2,5,8,-tetramethylchroman-2-carboxylic acid and the results are expressed as nmol/mg of protein (myocardial tissue) or mmol/l (plasma).

Biochemical Indicators of Oxidative Stress

Two damaging processes that occur due to ROS are peroxidative damage to lipids and oxidation of proteins. To determine the extent of both lipid peroxidation and protein oxidation in the myocardium after I-R, the following methods were used.

Lipid peroxidation measurements

To determine the amount of radical-mediated oxidative damage in the heart, left ventricular levels of two by-products of lipid peroxidation were measured. Lipid hydroperoxides were quantified using the ferrous oxidation/xylenol orange technique reported by Hermes-Lima (102). Briefly, following tissue homogenization in methanol, the membrane peroxides are mixed in solution with an iron source ($FeSO_4$), an acid (H_2SO_4) and a reactive dye (xylenol orange). In this mixture, the membrane peroxides oxidize Fe^{2+} to Fe^{3+} and the peroxides are reduced. The ionized acid assists in the reduction. The Fe^{3+} then reacts with the xylenol orange to form a Fe^{3+}-xylenol orange complex. Originally yellow in dye form, the Fe^{3+}-xylenol complex changes to a purplish color that can be detected spectrophotometrically at 580nm. Cumene hydroperoxide was used to generate a standard curve the standard for this assay.

In addition to lipid hydroperoxides, total 8-isoprostane was quantified by competitive enzyme immunoassay as per manufacturer's instruction (Cayman Chemical, Ann Arbor, MI). Measurement of 8-isoprostane, a member of the eicosanoid family, is based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase conjugate for a limited amount of the 8-isoprostane polyclonal antiserum. Briefly, following homogenization in methanol, samples were extracted and hydrolyzed via incubations in 100% ethanol and 15% potassium hydroxide. Resultant samples were then purified on a methanol-activated Sep-Pak C18 column and evaporated to dryness using a Supelco (Bellefonte, PA) vacuum centrifugation system. The remaining residue was then redissolved in EIA buffer, placed in microplates coated with antibody, and mixed with antiserum to 8-isoprostane and 8-isoprostane linked to acetylcholinesterase. Following a 24-hour incubation, wells were washed free of unbound reagents and Ellman's reagent was added to facilitate spectrophotometric measurement at 412nm. Standard curves constructed using known concentrations of 8-isoprostane were used to quantify total 8-isoprostane concentration in the samples.

Protein oxidation

I-R-induced myocardial protein oxidation was assessed using two methods. First, left ventricular protein thiols were measured spectrophotometrically as described by Jocelyn (115). Briefly, this assay measures the quantity and proportion of sulfhydryl groups (-SH) within the tissue compartments. The total amount of cellular thiols were determined by mixing DTNB (a sulfhydryl reagent that detects thiols) with tissue homogenate and quantifying spectrophotometrically at 412nm. DTNB was then reacted with homogenate following acid precipitation of proteins. Protein-bound thiol content was obtained mathematically by subtracting the non-protein bound thiol concentration from the total thiol concentration.

In addition to measuring thiols, total protein carbonyl content was assayed spectrophotometrically as described by Reznick and Packer (207), with modifications reported by Yan et al. (261). Tissue protein was extracted in a protease-treated 0.1 M phosphate buffer inhibitor (0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 40 μg/ml phenylmethylsulfonylfluoride. 1 mM EDTA), pH 7.4. DNA was removed from samples by treatment with 1 % streptomycin. The samples were then treated with 1 mM 2.4 dinitrophenylhydrazine (DNPH). The pellets were sonicated and washed three times in an ethyl acetate-ethanol solution (1:1 vol/vol). Following the final wash, pellets were dissolved (using sonication) in 6 M guanidine hydrochloride, pH 2.3. Tissue protein carbonyl content was quantified spectrophotometrically at 370 nm (extinction coefficient 22,000 $l \cdot mol^{-1} cm^{-1}$) and normalized to protein concentration as measured at 280 nm.

Model Justification

The Sprague Dawley rat was chosen as an experimental model for the following reasons: (1) the nature of these invasive experiments prevents the use of human subjects; (2) the Sprague Dawley rat is highly inbred and does not display large inter-animal variation in coronary collateral circulation (48,85); (3) the Sprague Dawley rat is an accepted model for the study of myocardial adaptations (114,137,186,197); and (4) this laboratory has had extensive experience with this strain. Because there is no evidence that gender differences exist in myocardial responses to I-R, exercise training, or to dietary antioxidant supplementation, the female Sprague Dawley rat was chosen based on experience with this gender (110,114,137). The rats were five months old at the time of sacrifice, an age representative of a young adult animal (159).

The antioxidant diet was chosen to provide global antioxidant protection in both aqueous and lipid phases of cells. Specific doses of antioxidants in the experimental diet were selected based on work of others demonstrating improvements in antioxidant capacity in a variety of rodent tissues (37-41,127,185).

Because myocardial stunning and myocardial infarction are distinct injuries and remains unknown whether the two conditions share common mechanisms, two separate *in vivo* I-R protocols were employed in these studies (17-21). A 20-minute ischemic period followed by 30 minutes of reperfusion was chosen as a model of myocardial stunning and a 60-minute ischemic period followed by two hours of reperfusion was selected to investigate the effects of the interventions on infarction (21). The *in vivo* surgical procedure used in these experiments has been used successfully by numerous investigators and has been shown to result in both regional myocardial ischemia and reperfusion (259-260). We believe that the inter-animal differences inherent to *in vivo* surgical techniques are clinically relevant and probably better reflect the types of I-R insults that occur in humans than do *in vitro* models.

Statistical Analyses

All dependent measures (contractile parameters, antioxidant concentrations, enzyme activities, markers of oxidative damage, and HSP 72/73 accumulation) were subjected to two-way analyses of variance. When appropriate, *post hoc* analyses were applied. Significance was established *a priori* at $p<0.05$.

CHAPTER 4
RESULTS

Overview of Experimental Findings

These experiments examined the individual and combined effects of dietary supplementation with antioxidants and short-term exercise training on myocardial performance and biochemical responses during *in vivo* I-R in the adult rat. The major findings of the study were that exercise training resulted in improved cardiac performance during both ischemia and reperfusion regardless of diet (figure 2). Antioxidant supplementation did not have a detrimental effect on the cardioprotection associated with exercise despite an associated attenuation of HSP72 (figure 3). In addition to improved cardiac performance during a relatively short "stunning" protocol, exercise training, independent of diet, protected the heart against myocardial infarction following a long-term I-R protocol (figure 4). Supplementation with antioxidants, however, provided superior protection against lipid peroxidation and protein oxidation following short-term I-R (figure 5) compared with exercise training alone. Details of experimental findings are outlined in the following sections.

Antioxidant Intake

Animals tolerated the antioxidant-supplemented diet well and no differences in food intake existed between the control diet and antioxidant diet groups. Antioxidant intake is summarized in Table 2.

Table 2. Food and antioxidant intake

Group:	**Control Untrained**	**Control Trained**	**Antioxidant Untrained**	**Antioxidant Trained**
Avg. body wt (g)	291	280	303	288
Avg. food intake (g/day)	15.2	14.7	15.0	15.0
Vitamin E (IU/day)	1.14	1.07	14.90	15.17
Selenium (mg/day)	0.0004	0.0004	0.0045	0.0045
Vitamin C (mg/day)	0	0	14.90	15.17
β-carotene (mg/day)	0	0	3.35	3.41
N-acetyl cysteine (mg/day)	0	0	2.98	3.03
Catechin (mg/day)	0	0	1.49	1.52
Coenzyme Q_{10} (mg/day)	0	0	0.45	0.45

Myocardial Contractile Function

Myocardial contractile performance was assessed throughout an *in vivo* I-R "stunning" protocol utilizing a fluid filled carotid catheter advanced into the left ventricle. The impact of individual and combined treatment with antioxidants and exercise training on left ventricular developed pressure during the 20-minute ischemia/ 30-minute reperfusion protocol is illustrated in figure 2. Left ventricular developed pressure (LVDP) was significantly ($p<0.05$) higher at two and five minutes of ischemia in the exercise-trained groups compared to sedentary groups regardless of diet. Trained animals fed a control diet maintained higher ($p<0.05$) LVDP throughout reperfusion compared to untrained animals fed a control diet; by minute three of reperfusion, the antioxidant-supplemented/trained group also achieved significantly ($p<0.05$) higher LVDP compared to sedentary controls. By minute 10 of reperfusion, antioxidant-supplemented/trained groups had higher ($p<0.05$) LVDP than the untrained counterparts. Although LVDP tended to be higher in the antioxidant supplemented/ untrained animals compared to the control diet/untrained group, these differences were not significant ($p>0.05$)

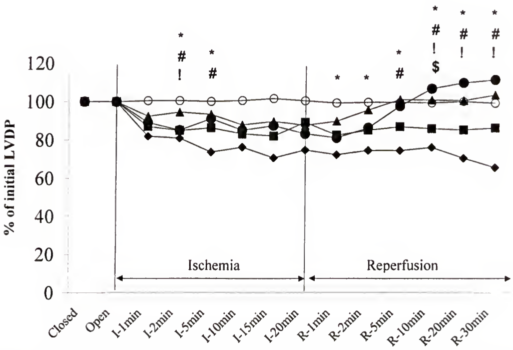

Figure 2. Left Ventricular Developed Pressure (20 min Ischemia/30 min Reperfusion); open circles (○) = sham surgery; filled diamonds (◆) = control diet/untrained; filled triangles (▲) = control diet/trained; filled squares (■) = antioxidant diet/untrained; filled circles (●) = antioxidant diet/trained. * = control diet/untrained significantly different than control diet trained; # = control diet/untrained significantly different than antioxidant diet/trained; ! = antioxidant diet/untrained significantly different than antioxidant diet/trained; $ = antioxidant diet/untrained significantly different than control diet/trained ($p<0.05$).

Myocardial Infarction

Myocardial infarction was assessed following 60 minutes of ischemia and 120 minutes of reperfusion using the TTC staining technique. No significant differences existed between groups with respect to area at risk for infarction (figure 3). As shown in figure 4, control diet/untrained animals had a significantly greater infarction area than all other groups (p<0.05). No difference existed between the remaining three groups (p>0.05). Representative heart sections stained for area at risk and for infarction are shown in figure 5.

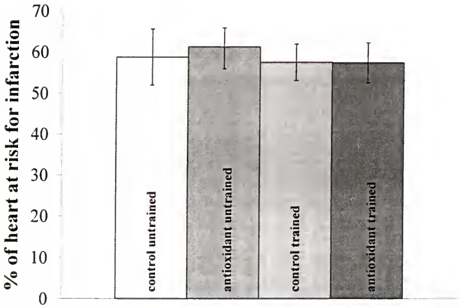

Figure 3. Risk Area expressed as a percentage of the heart; values are means ± SEM; no significant differences among groups (p>0.05).

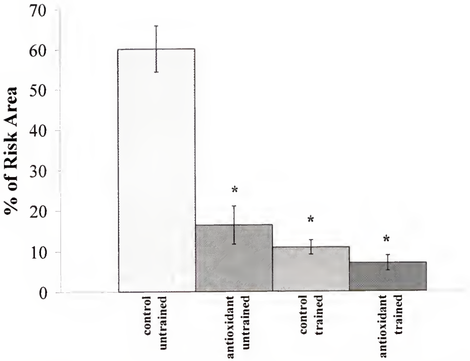

Figure 4. Infarction area expressed as a percentage of the area at risk; values are means ± SEM; * indicates less than control untrained ($p<0.05$).

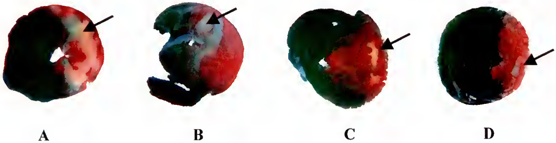

Figure 5. Representative heart sections stained for infarction following long-term I-R. Blue stained areas represent tissues that were well perfused during coronary occlusion. Brick red areas represent tissue that, despite ischemia, was not infarcted. Yellowish-white areas represent infarcted tissue (indicated by arrows). A = control diet/untrained; B = antioxidant diet/untrained; C = control diet/trained; D = antioxidant diet/trained.

Myocardial HSP72 Accumulation

Portions of the left ventricular free wall were used for quantification of HSP72 protein accumulation. Results, summarized in figure 6, demonstrate a significantly greater ($p<0.05$) accumulation of HSP72 following exercise training in the control diet fed animals compared to untrained groups. However, exercise training did not result in significant increases in HSP72 accumulation in antioxidant supplemented animals ($p>0.05$). No differences were observed in the accumulation of the constitutive isoform HSP73 (data not shown).

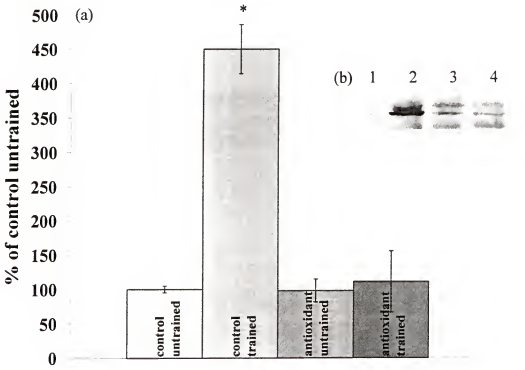

Figure 6. Left ventricular HSP72. (a) Histogram comparing densitometric analyses of all western blots; values are means ± SEM; * indicates greater than all other groups ($p<0.05$); (b) Representative western blot for HSP72; Lane 1 = control diet untrained; Lane 2 = control diet trained; Lane 3 = antioxidant diet untrained; Lane 4 = antioxidant diet trained.

Total Antioxidant Capacity

To evaluate the effect of experimental interventions (diet and activity) on the global antioxidant capacity of heart tissue, the Total Antioxidant Status (TAS) kit was employed. TAS was assayed in plasma (figure 7a) and left ventricular tissue samples (figure 7b) from sham surgery animals only and, therefore, does not reflect the impact of ischemia or reperfusion. Antioxidant diet-fed animals had significantly higher ($p<0.05$) TAS compared to control diet-fed rats. Interestingly, exercise training resulted in a decrease in plasma TAS ($p<0.05$).

Myocardial Antioxidant Enzyme Activity

Table 3 contains mean (± SEM) activities of myocardial glutathione peroxidase (GPX), total superoxide dismutase (SOD), manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (Cu-ZnSOD), and catalase (CAT). Antioxidant enzyme activities were assayed in left ventricular tissues from sham surgery animals and, therefore, do not reflect the impact of ischemia or reperfusion on enzyme activities. Total and MnSOD activity was higher in both exercise-trained groups, regardless of diet, compared to sedentary groups ($p<0.05$). While no differences in CAT activity existed between groups, GPX activity was significantly higher in the antioxidant/trained group compared to all other groups ($p<0.05$).

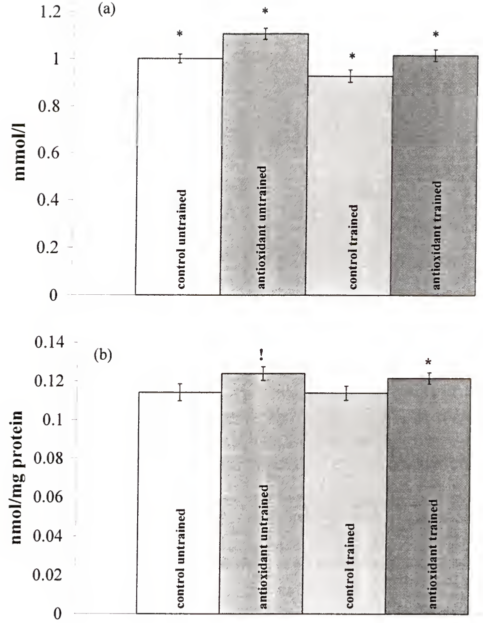

Figure 7. Assessment of total antioxidant status; (a) Total antioxidant status of plasma; values are means ± SEM; * indicates different than all other groups ($p<0.05$); (b) Total antioxidant status of left ventricular tissues; values are means ± SEM; * indicates different than control trained and control untrained ($p<0.05$); ! indicates different than control untrained ($p=0.059$).

Table 3. Summary of the effects of exercise training and antioxidant supplementation on endogenous antioxidant enzyme activity (values are means ± SEM)

	Control Diet		**Antioxidant Diet**	
Enzyme (activity per mg protein)	**Untrained** (n=11)	**Trained** (n=11)	**Untrained** (n=11)	**Trained** (n=10)
GPX (μmol/min)	0.255±0.007	0.247±0.020	0.217±0.011	0.305±0.018[b]
Total SOD (U)	45.85±1.18	54.89±3.21[a]	45.53±2.42	55.39±6.10[a]
MnSOD (U)	32.73±0.98	37.06±2.30[a]	31.64±2.11	39.38±4.98[a]
CuZnSOD (U)	13.12±1.69	17.83±3.68	13.89±2.38	16.01±2.61
CAT (U)	1.79±0.14	1.66±0.08	1.49±0.16	1.69±0.16

a = different than both untrained groups ($p<0.05$)
b = different than all other groups ($p<0.05$)

Lipid Peroxidation

Two markers of lipid peroxidation were used to determine the effects of exercise and antioxidants on left ventricular damage resulting from the short-term I-R exposure. Figures 8a and 8b contain the mean (± SEM) values for myocardial lipid hydroperoxides and 8-isoprostane concentrations in all experimental groups, respectively. A brief summary of the major findings is as follows. First, note that these two markers of lipid peroxidation follow similar trends with lipid peroxidation being less in all antioxidant-supplemented groups compared to control diet-fed groups ($p<0.05$). However, only the assay of 8-isoprostanes detected significant elevations in lipid peroxidation resulting from I-R. Antioxidant supplementation resulted in protection of the left ventricular myocardium from this I-R-induced lipid peroxidation ($p<0.05$).

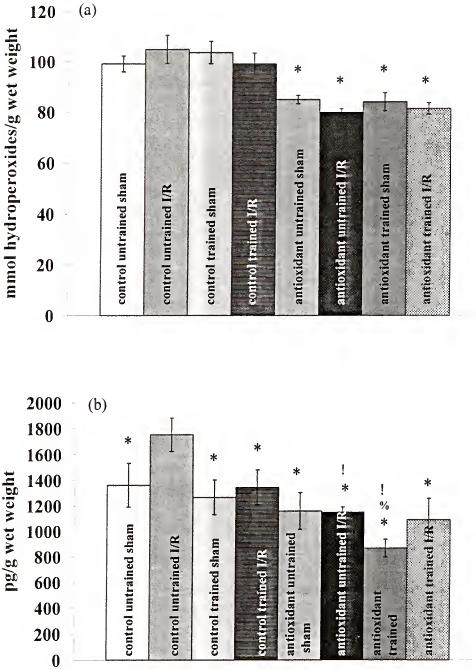

Figure 8. Left ventricular lipid peroxidation; (a) Lipid hydroperoxide concentration; * indicates less than all control diet groups ($p<0.05$) (b) 8-isoprostane concentration; * indicates less than control untrained I/R; ! less than control untrained and trained sham; % less than control trained I/R and less than antioxidant untrained sham and I/R ($p<0.05$).

Protein Oxidation

Protein thiols (figure 9) and protein carbonyls (figure 10) were assayed as measures of protein oxidation in the myocardium of all experimental groups. Myocardial total and protein thiol concentrations were significantly lower ($p<0.05$) in all I-R surgery groups compared to all sham surgery groups. While these results are indicative of I-R-induced oxidative stress, no differences in this measure of protein oxidation were evident with either antioxidant supplementation or exercise training. Protein carbonyl concentration was lower in all antioxidant-supplemented groups compared to control diet groups ($p<0.05$).

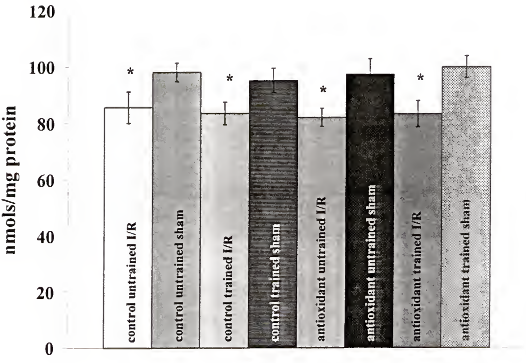

Figure 9. Left ventricular protein thiol concentration; * indicates less than the respective sham group ($p<0.05$).

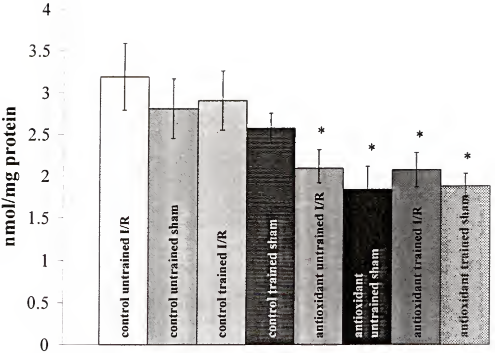

Figure 10. Left ventricular protein carbonyl concentration; * indicates less than all control diet groups ($p<0.05$).

CHAPTER 5
DISCUSSION

Overview of Principal Findings

This is the first experiment to examine the combined effects of exercise training and antioxidant supplementation on myocardial contractile performance and biochemistry during *in vivo* I-R in adult rats. A major finding of this study was in support of our first hypothesis that HSP72 would be attenuated following the combined intervention of exercise training and antioxidant supplementation compared to exercise training alone. This attenuation of HSP72, however, did not decrease the exercise-associated improvements in contractile performance during a short-term "stunning" I-R protocol. Further, exercise training and antioxidant supplementation, individually but not synergistically, provided protection against infarction resulting from long-term I-R. These two findings did not support our hypotheses which were based on the notion that the attenuation of HSP72, resulting from concurrent antioxidant supplementation and exercise training, would decrease the exercise-associated cardioprotection. The final finding was that exercise training increased the activity of the enzymatic antioxidant superoxide dismutase while antioxidant supplementation resulted in an improved global antioxidant capacity and protected the myocardium against I-R-induced lipid peroxidation and protein oxidation. Details of these findings follow.

Exercise-Associated Cardioprotection and HSP72

The current data confirm previous work from this laboratory with endurance exercise training resulting in both an increase in myocardial HSP72 accumulation and an associated cardioprotection during short-duration *in vivo* I-R (53-55,198). While transfection and transgenic models demonstrating the relationship between HSP72 overexpression and protection against I-R-induced myocardial injury are convincing, new evidence suggesting that HSP72 is not essential for exercise-associated cardioprotection is compelling. Taylor and colleagues recently demonstrated that exercise in that absence of HSP72 accumulation results in improved cardiac performance during an *in vitro* model of I-R (235). We have recently confirmed these results using an *in vivo* model of I-R and have, additionally, found no compensatory increases in HSP10, 27, 40, 60, 73, or 90 in the absence of HSP72 despite a perseverance of the exercise-associated cardioprotection (91). The findings of these experiments contribute to the accumulating notion that HSP72 may not be mandatory for exercise training to confer cardioprotection. That is, exercise in conjunction with antioxidant supplementation resulted in a significant attenuation of HSP72 accumulation but did not result in an attenuation of cardioprotection during *in vivo* I-R.

By demonstrating that antioxidant supplementation attenuates the exercise-associated accumulation of HSP72, these experiments also strongly suggest that production of ROS during exercise plays an important role in the induction of HSP72. While the specific stress responsible for HSP induction remains unknown, it has been speculated that a host of physiochemical stresses associated with exercise, including (1) increases in temperature or calcium concentration; (2) decreases in pH or oxygen content; (3) exposure to oxidants, or (4) any other stresses capable of damaging protein, may contribute to the exercise-induced HSP72 accumulation (147). Indeed, reactive oxygen species have been found to play an important role in the activation of heat shock factor 1 (HSF1) and the appearance of increased amounts of HSP72 mRNA (13,130,184). Further, recent reports that exercise, in the absence of an increase in colonic temperature, results in cardioprotection but no accumulation of myocardial HSP72 suggest that an increase in body temperature is mandatory (91,235). However, the current experiments imply that exercise may involve a redundant signaling pathway for HSP72 induction. That is, enhancing antioxidant capacity attenuated HSP72 accumulation despite a rise in body temperature. Clearly, further work is required to describe the cellular signals involved in the exercise-induced expression of HSP72.

Exercise and Antioxidants Attenuate Infarction Size

Current evidence supports a role for oxidative stress in the damage leading to myocardial infarction. Many have demonstrated that enhancing antioxidant capacity attenuates myocardial infarction size following I-R (105,138,165,236). However, not all studies have led to the same conclusion; several investigations have found that enhancing cellular enzymatic antioxidant defenses has no effect on infarction size (232,242,245). The current investigation revealed a positive effect of a comprehensive antioxidant supplementation protocol on the infarction resulting from a relatively severe regional ischemic bout (60 minutes) followed by two hours of reperfusion. Exercise training alone also decreased myocardial infarction size following I-R but was not significantly different than antioxidant supplementation alone. Indeed, exercise, along with other stresses capable of inducing HSP72, has been shown to result in smaller infarction size (12,157,260). However, the combination of short-term exercise training and antioxidant supplementation did not have either an additive effect in protecting the left ventricle from infarction resulting from I-R, nor did the antioxidant-associated attenuation of HSP72 result in attenuated protection against infarction. Similar to the findings in the short-duration I-R experiments, this finding contributes to the notion that an accumulation of HSP72 may not be mandatory for exercise training to confer cardioprotection. Perhaps only basal amounts of HSP72 are required to protect against myocardial infarction and the exercise-induced increases in SOD activity may be the chief mechanism of protection against I-R-induced injury (91,260).

Antioxidant Supplementation and Myocardial Antioxidant Capacity

The antioxidant diet utilized in these experiments was modeled after diets used by others who demonstrated protection of a variety of tissues against oxidative injury (37-41,127,185). The supplementation protocol used in this study resulted in an enhanced total antioxidant capacity of both plasma and left ventricular tissues. Given the reported redox regulation of the mitochondrial isoform of SOD, it is logical to hypothesize that antioxidant supplementation might result in a decrease in SOD activity (244). Antioxidant supplementation in this study, however, resulted in no change in the activity of the primary antioxidant enzymes assayed. While a few reports of increased SOD activity following antioxidant supplementation exist (182-183), our findings are in agreement with the majority of existing literature (27,34,184).

Myocardial Lipid Peroxidation

Lipid peroxidation, capable of altering membrane fluidity and permeability, is one of the most damaging processes that occurs to the myocardium during I-R. Even small changes in the structure of the phospholipid membrane structure have been to interfere with normal selective permeability of cell membranes (158). For example, lipid peroxidation of the sarcoplasmic reticulum membrane can result in altered calcium handling and subsequent contractile dysfunction during I-R (212). Lipid peroxidation may also cause disturbances in oxidative metabolism. Oxygen radical-mediated loss of cardiolipin content from mitochondrial membranes has been proposed to be the cause of disruptions in cytochrome c oxidase activity and alterations in oxidative metabolism in the post-ischemic, reperfused heart (190).

In this study, two measures of lipid peroxidation were used to determine if dietary supplementation decreased left ventricular oxidative damage following I-R. Both assays revealed less lipid peroxidation in all antioxidant-supplemented groups compared to control diet-fed groups. However, only the ELISA assay of 8-isoprostanes detected increases in lipid peroxidation following I-R compared with sham surgery. Antioxidant supplementation, but not exercise training, resulted in protection of the left ventricular myocardium from this I-R-induced lipid peroxidation. These findings are in agreement with studies that have reported decreased *in vitro* lipid peroxidation following antioxidant supplementation (37,39,127,185). Importantly, the current study demonstrates that this dietary antioxidant regimen decreases I-R induced lipid peroxidation *in vivo*. Others have, accordingly, shown that important "networking" antioxidants such as ascorbic acid, vitamin E, glutathione, and ubiquinones are depleted during I-R and in the presence of ROS (95). Thus, supplementation protocols such as the one used in these experiments may help prevent this selective depletion.

Finally, these data may provide information regarding the utility of these two widely used assays for detecting *in vivo* lipid peroxidation. Because lipid peroxidation is a complex process occurring in multiple stages, each of the many techniques available for measuring the rate of peroxidation measures something different and no one method, by itself, can be said to be an accurate overall measure. While the assay of hydroperoxides gives an estimation of peroxidation in the early stages, one must bear in mind that this measurement is highly dependent on the rate of initiation of peroxidation and on how rapidly peroxides are decomposing to other products in the lipid peroxidation pathway (90). F2-isoprostanes, on the other hand, arise primarily by peroxidation of phospholipid containing arachidonic acid (90) and may be a more stable product to assay than the rapidly removed peroxides. Indeed, it is important to employ more than one marker of lipid peroxidation to help accurately describe the oxidative damage to cellular lipids.

Myocardial Protein Oxidation

Oxidative damage to proteins can lead to functional alterations in receptors, signaling pathways, enzymes, membrane channels, and transport proteins; further secondary damage to other biomolecules can occur as a result of protein damage (i.e., via damage to DNA repair enzymes or polymerases). Unfortunately, measuring oxidative damage to proteins remains a difficult task (90). The two general markers of protein oxidation employed in these studies, protein thiols and protein carbonyls, produced somewhat different results. As expected, myocardial protein thiol concentrations were significantly higher in all sham surgery groups compared to I-R groups. While these results are indicative of I-R-induced oxidative stress, no differences in this measure of protein oxidation were evident with either antioxidant supplementation or exercise training. In contrast, protein carbonyl concentration was lower in all antioxidant-supplemented groups compared to control diet groups. However, due to greater variability within the groups, only statistical trends existed between sham and I-R surgery groups.

The role of HSPs in prevention of protein oxidation, repair of damaged proteins, and facilitation of proteolysis of damaged proteins is a topic of much interest. Indeed, HSP72 appears to play a role in preventing irreversible protein damage during myocardial I-R and could, theoretically, result in protection against formation of carbonyls or loss of thiol groups in exercise-trained rats (12-13,179). Similarly, exercise-associated increases in SOD activity could also provide protection against protein oxidation. However, in this study, exercise training did not result in protection of cellular proteins against I-R-induced protein oxidation despite an increase in HSP72 accumulation (control diet fed/exercise-trained group) and SOD activity. Further, very similar antioxidant supplementation protocols have resulted in decreases in heme protein oxidation during *in vitro* oxidative challenges (37,39,127). In this study, antioxidant supplementation increased total antioxidant status and resulted in lower levels of protein carbonyls in the myocardium *in vivo*.

The explanation for the disparity in our measures of protein oxidation is difficult to provide. Others have reported variability with measurement of protein carbonyls, similar to that seen in this study, likely arising from the multiple washing steps necessary to eliminate unbound DNPH (207). Indeed, our results emphasize the importance of utilizing several measures when assessing protein oxidation. Further, while others have shown that antioxidant diets similar to the one employed here have resulted in decreases in heme protein oxidation during *in vitro* oxidative challenges, the protection of tissues against this protein oxidation was dependent on the type of radical generating system employed (37,39,127). This is the first investigation of the impact of a comprehensive antioxidant supplementation protocol on protein oxidation resulting from an *in vivo* oxidative event. It is possible that the protection against the physiological oxidative challenge imposed by I-R, compared to an nonphysiological *in vitro* challenge, may have been too small to detect with both of the techniques employed in this study. Another possible explanation is that markers of protein oxidation may have been impacted by reperfusion length. This notion was supported by Poston and Parenteau (1992) who found that carbonyl levels rose during myocardial ischemia and were greater than four fold higher than initial values at five minutes of reperfusion but, with continued reperfusion, declined to only 150% of initial values at 15 minutes of reperfusion (196). Finally, while care was taken to prevent *ex vivo* oxidation (i.e., rinsing in an antioxidant buffer and rapidly freezing in liquid nitrogen), tissue storage and handling can impact tissue oxidation status, particularly with respect to thiol groups (90).

Possible Mechanisms of Exercise-Associated Cardioprotection

The mechanisms for exercise-induced cardioprotection during myocardial I-R in the absence of HSP72 remain speculative. One possibility is that exercise-induced cardioprotection is related to an improvement in antioxidant capacity. Indeed, training-induced alterations in antioxidant capacity appear to be tissue and muscle fiber type specific (111,134). The results of this study were consistent with previous reports from our laboratory and others, with exercise training resulting in a significant increase in the activity of MnSOD (52,91,197-198,260). Increases in the activity of MnSOD activity, resulting either from whole-body hyperthermia, exercise training, or overexpression in a transgenic mouse model, has been implicated in providing protection against myocardial I-R injury (38,108,259-260). Thus, the observed increase in SOD activity in both exercise-trained groups in this study could be responsible for the improved myocardial contractile performance that persisted even when HSP72 was attenuated.

Another possible mechanism for exercise-related cardioprotection is an associated increase in myocardial glycolytic capacity. This change in bioenergetics could translate to an enhanced supply of ATP to compensate for the decrease in oxidative phosphorylation that occurs during myocardial ischemia. It is unlikely, however, that as few as three to five days of training could lead to such changes in energetics.

Exercise training could also result in increased myocardial vascularity, an adaptation that would assist in maintaining ventricular blood flow during ligation of the coronary arteries and permit ATP production via oxidative phosphorylation during ischemia. This vascular adaptation remains controversial and, like changes in glycolytic capacity, is unlikely to occur with short-term training. Another possible exercise-related adaptation is a longer diastolic period which, if coupled with increased vascularity, could allow greater perfusion of the myocardium due to the longer period of coronary blood flow (14).

As reported in these experiments, exercise training results in a higher –dP/dt which is a reflection of calcium re-uptake into the sarcoplasmic reticulum. This accelerated calcium re-uptake is correlated with a more rapid ventricular relaxation and a correspondingly longer diastolic period. This training-induced change in calcium handling is related to expression of the sarcoplasmic reticulum calcium pump. Activity of the SR calcium pump is highly correlated with Ca^{++}-ATPase activity which has been shown to increase in the myocardium following exercise training (234). Functionally, this enzymatic adaptation could translate to an increase in ventricular diastole.

As demonstrated by these experiments and others, exercise training results in enhanced post-ischemic left ventricular contractile recovery. This improved ventricular contractile performance likely involves a decrease in myocardial injury during I-R due to any combination of the following: (1) greater high-energy phosphate shuttling, (2) enhanced calcium handling and inotropic responsiveness to external calcium, and (3) decreased diastolic stiffness. Exercise may protect against myocardial I-R injury by decreasing the magnitude of oxidant damage (19,62,111). Oxidants are produced in the myocardium during reperfusion and administration of radical scavengers attenuates myocardial stunning (19). It is hypothesized that myocardial stunning is due, at least in part, to oxidation of calcium handling proteins within the sarcoplasmic reticulum (19,204-205).

Summary and Conclusions

This is the first experiment to examine the combined effects of exercise training and antioxidant supplementation on myocardial contractile performance and biochemistry during *in vivo* I-R in adult rats. Primary findings include the following: (1) exercise-associated increases in HSP72 accumulation are attenuated by dietary antioxidant supplementation; (2) attenuation of HSP72 did not decrease the exercise-associated improvements in contractile performance during a short-term “stunning” I-R protocol; (3) evidence of additive effects of antioxidant supplementation and exercise training did not exist with regard to *in vivo* contractile performance; (4) exercise training and antioxidant supplementation provided individual, but not additive, protection against infarction following relatively long-term I-R; and (5) exercise training increased SOD activity while antioxidant supplementation resulted in an improved global antioxidant capacity and protected the myocardium against I-R-induced lipid peroxidation and protein oxidation without altering antioxidant enzyme activity. Future research efforts should be directed at elucidating the mechanisms for exercise-induced cardioprotection during myocardial I-R in the absence of HSP72.

REFERENCES

1. Abravaya, K., M.P. Myers, S.P. Murphy, and R.I. Morimoto. The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. *Genes and Development* 6: 1153-1164, 1992.

2. Aebi, H. Catalase in vitro. *Methods in Enzymology* 105: 121-126, 1984.

3. Aberg, F., E.L. Appelkvist, G. Dallner, and L. Ernster. Distribution and redox state of ubiquinones in rat and human tissues. *Archives in Biochemistry and Biophysics* 295(2): 230-234, 1992.

4. Alessio, H.M. Lipid peroxidation processes in healthy and diseased models. In: *Exercise and Oxygen Toxicity*, edited by C.K. Sen, L. Packer, and O. Hanninen. Amsterdam: Elsevier Science, 1994, p. 269-295.

5. Alessio, H.M., A.H. Goldfarb, and G. Cao. Exercise-induced oxidative stress before and after vitamin C supplementation. *International Journal of Sports Nutrition* 7(1): 1-9, 1997.

6. Angelidis, C.E., I. Lazaridis, and G.N. Pagoulatos. Constitutive expression of heat-shock protein 70 in mammalian cells confers thermoresistance. *European Journal of Biochemistry* 199: 35-39, 1991.

7. Barry, W.H. Mechanisms of myocardial cell injury during ischemia and reperfusion. *Journal of Cardiac Surgery* 2: 375-383, 1987.

8. Barsacchi, R, M. Coassin, M. Majorino, M. Pelosi, G. Simonelli, and F. Ursini. Increased ultra weak chemiluminescence from rat heart at postischemic reoxygenation: protective role of vitamin E. *Free Radical Biology and Medicine* 6: 573-579, 1989.

9. Beckmann, R.P., L.A. Mizzen, and W.J. Welch. Interaction of HSP70 with newly synthesized proteins: Implications for protein folding and assembly. *Science* 248: 850-854, 1990.

10. Bendich, A. The safety of beta-carotene. *Nutrition and Cancer* 11(4): 207-214, 1988.

11. Benjamin, I.J., S. Horie, M.L. Greenberg, R.J. Alpern, and R.S. Williams. Induction of stress proteins in cultured myogenic cells. *Journal of Clinical Investigation* 89: 1685-1689, 1992.

12. Benjamin, I.J. and D.R. McMillan. Stress (heat shock) proteins Molecular chaperones in cardiovascular biology and disease. *Circulation Research* 83: 117-132, 1998.

13. Benjamin, I.J. and R.S. Williams. Expression and function of stress proteins in the ischemic heart. In: *The Biology of Heat Shock Proteins and Molecular Chaperones,* edited by R.I. Morimoto, A. Tissieres, and C. Georgopoulos. Cold Spring Harbor: Cold Spring Harbor: Laboratory Press, 1994, p. 533-553.

14. Bersohn, M. and J. Scheuer. Effect of ischemia on the performance of hearts from physically trained rats. *American Journal of Physiology* 20: H215-H218, 1978.

15. Bjorneboe, A., G. Bjorneboe, and C.A. Devron. Absorption, transport and distribution of vitamin E. *Journal of Nutrition* 120: 233-242, 1990.

16. Blebea, J., J.C. Kerr, J.Z. Shumko, R.N. Feinberg, and R.W. Hobson. Quantitative histochemical evaluation of skeletal muscle ischemia and reperfusion injury. *Journal of Surgical Research* 43: 311-321, 1987.

17. Bolli, R., B. Patel, M. Jeroudi, E. Lai, and P. McCay. Demonstration of free radical generation in "stunned myocardium" of intact dogs with the use of the spin trap alpha-phenyl N-tert-butyl nitrone. *Journal of Clinical Investigation* 82: 476-485, 1988.

18. Bolli, R., W.X. Zhu, C.J. Hartley, L.H. Michael, J.E. Repine, M.L. Hess, R.C. Kukreja, and R. Roberts. Attenuation of dysfunction in the postischemic "stunned" myocardium by dimethylthiourea. *Circulation* 76: 458-468, 1987.

19. Bolli, R. Oxygen-derived free radicals and post-ischemic myocardial dysfunction “stunned myocardium.” *Journal of the American College of Cardiology* 12: 239-249, 1988.

20. Bolli, R., M.O. Jeroudi, and B.S. Patel. Direct evidence that oxygen-derived free radicals contribute to post-ischemic myocardial dysfunction in the intact dog. *Proceedings of the National Academy of Sciences USA* 86: 4695-4699, 1989.

21. Bolli, R. and E. Marban. Molecular and cellular mechanisms of myocardial stunning. *Physiological Reviews* 79: 609-634, 1999.

22. Booth, F. and D.B. Thomason. Molecular and cellular adaptation of muscle in response to exercise: perspectives of various models. *Physiological Reviews* 71: 541-585, 1991.

23. Bors, W., W. Heller, C. Michel, and M. Saran. Flavonoids as antioxidants: determination of radical-scavenging efficiencies. *Methods in Enzymology* 186: 343-355, 1990.

24. Bowles, D., R. Farrar, and J. Starnes. Exercise training improves cardiac function after ischemia in the isolated, working rat heart. *American Journal of Physiology* 263: H804-H809, 1992.

25. Bradford, A. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254, 1976.

26. Bradford, N.B., M. Fina, I.J. Benjamin, R.W. Moredith, K.H. Graves, P. Zhao, S. Gavva, A. Wiethoff, A.D. Sherry, C.R. Malloy, and R.S. Williams. Cardioprotective effects of 70-kDa heat shock protein in transgenic mice. *Proceedings of the National Academy of Science USA* 93: 2339-2342, 1996.

27. Buckman T.D., M.S. Sutphin, and C.D. Eckhert. A comparison of the effects of dietary selenium on selenoprotein expression in rat brain and liver. *Biochimica et Biophysica Acta* 1163(2): 176-184, 1993.

28. Burton, G.W. Antioxidant action of carotenoids. *Journal of Nutrition* 119(1): 109-111, 1989.

29. Burton, G.W. and K.U. Ingold. Autooxidation of biological molecules: I. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidant in vitro. *Journal of the American Chemical Society* 103: 6472-6477, 1981.

30. Burton, G.W. and K.U. Ingold. beta-Carotene: an unusual type of lipid antioxidant. *Science* 224(4649): 569-573, 1984.

31. Burton, G. and M. Traber. Vitamin E: antioxidant activity, biokinetics, and bioavailability. *Annual Reviews in Nutrition* 10: 357-382, 1990.

32. Cao, G., E. Sofic, and R.L. Prior. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radical Biology and Medicine* 22(5): 749-760, 1997.

33. Carlson, N., S. Rogers, and M. Rechsteiner. Microinjection of ubiquitin: changes in protein degradation in HeLa cells subjected to heat-shock. *Journal of Cell Biology* 104: 547-555, 1987.

34. Castenmiller, J.J., S.T. Lauridsen, L.O. Dragsted, K.H. van het Hof, J.P. Linssen, and C.E. West. Beta-carotene does not change markers of enzymatic and nonenzymatic antioxidant activity inhuman blood. *Journal of Nutrition* 129(12): 2162-2169, 1999.

35. Chambers, D.L. and J.M. Downey. Xanthine oxidase, free radicals, and myocardial ischemia. In: *Handbook of Free Radicals and Antioxidants in Biomedicine*, edited by J. Miguel, A.T. Quintilla, and H. Webereds. Boca Raton: CRC Press, 1989, p. 263-273.

36. Chen, L.H. Interaction of vitamin E and ascorbic acid (review). *In Vivo* 3(3): 199-209, 1989.

37. Chen, H., L.J. Pellett, H.J. Andersen, and A.L. Tappel. Protection by vitamin E, selenium, and beta-carotene against oxidative damage in rat liver slices and homogenate. *Free Radical Biology and Medicine* 14: 473-482, 1993.

38. Chen, Z., B. Sui, Y-S. Ho, R. Vincent, C.C. Chua, R.C. Hamdy, and B.H.L. Chua. Overexpression of MnSOD protects against myocardial ischemia-reperfusion injury in transgenic mice. *Journal of Molecular and Cellular Cardiology* 30: 2281-2289, 1998.

39. Chen, H. and A. Tappel. Protection by vitamin E, selenium, trolox C, ascorbic acid palmitate, acetylcysteine, coenzyme Q, beta-carotene, and (+)- catechin against oxidative damage to liver slices measured by oxidized heme proteins. *Free Radical Biology and Medicine* 16(4): 437-444, 1994.

40. Chen, H. and A. Tappel. Protection of vitamin E, selenium, trolox C, ascorbic acid palmitate, acetylcysteine, coenzyme Q_0, coenzyme Q_{10}, beta-carotene, canthaxanthin, and (+)- catechin against oxidative damage to rat blood and tissues in vivo. *Free Radical Biology and Medicine* 18(5): 949-953, 1995.

41. Chen, H. and A. Tappel. Protection by multiple antioxidants against lipid peroxidation in rat liver homogenate. *Lipids* 31(1): 47-50, 1996.

42. Chiang, H.L., S. Terlecky, C. Plant, and J.F. Dice. A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science* 246: 382-385, 1989.

43. J.S.Coombes, S.K. Powers, H. Demirel, J. Jessup, H.K.Vincent, K.L. Hamilton, H. Naito, R.A. Shanely, C.K. Sen, L. Packer and L.L. Ji. Effect of combined supplementation with vitamin E and alpha-lipoic acid on myocardial performance during in vivo ischemia-reperfusion. *Acta Physiologica Scandinavica* (in press).

44. J.S.Coombes, S.K. Powers, H.A. Demirel, K.L. Hamilton, R.A. Shanely, H. K.Vincent and H. Naito. Vitamin E deficiency fails to effect myocardial performance during in vivo ischemia-reperfusion. *International Journal of Vitamin and Mineral Research* (in press).

45. Currie, R.W., M. Karmazyn, M. Kloc, and K. Mailer. Heat-shock response is associated with enhanced postischemic ventricular recovery. *Circulation Research* 63: 543-549, 1988.

46. Currie, R.W. and F.P. White. Trauma induced protein in rat tissues: a physiological role for a "heat shock" protein? *Science* 214: 72-73, 1981.

47. Currie, R.W., R.M. Tanguay, and J.G. Kingma. Heat-shock response and limitation of tissue necrosis during occlusion/reperfusion in rabbit hearts. *Circulation* 87: 963-971, 1993.

48. Curtis, M., B. Macleod, and M. Walker. Models for the study of arrhythmias in myocardial ischemia and infarction: the use of the rat. *Journal of Molecular and Cellular Cardiology* 19: 390-419, 1987.

49. Das, D.K. Naturally occurring flavonoids: Structure, chemistry, and high-performance liquid chromatography methods for separation and characterization. *Methods in Enzymology* 234: 410-420, 1994.

50. Das, D.K., R.M. Engelman, and Y. Kimura. Molecular adaptation of cellular defenses following preconditioning of the heart by repeated ischemia. *Cardiovascular Research* 27: 578-584, 1993.

51. Davies, T., J. Kelleher, C.L. Smith, and M.S. Losowsky. The effect of orotic acid on the absorption, transport and tissue distribution of alpha-tocopherol in the rat. *Internationale Zeitschrift Fur Vitaminforschung* 41: 360-365, 1971.

52. Demirel, H.A., S.K. Powers, C. Caillaud, J.S. Coombes, H. Naito, L.A. Fletcher, I. Vrabas, J.V. Jessup, and L.L. Ji. Exercise training reduces myocardial lipid peroxidation following ischemia reperfusion. *Medicine and Science in Sports and Exercise* 30: 1211-1216, 1998a.

53. Demirel, H.A., S.K. Powers, R.A. Shanely, A.M. Zergeroglu, J. Coombes, and K. Ward. Effects of heat stress and exercise on myocardial heat shock proteins and responses to ischemia reperfusion. *Federation of American Societies for Experimental Biology Journal* 12 (3): A2165, 1998c.

54. Demirel, H., S.K. Powers, J.S. Coombes, H. Naito, H.K. Vincent, R.A. Shanely, and K.L. Hamilton. Short-term endurance exercise training and myocardial ischemia-reperfusion injury in the rat. *Journal of Applied Physiology* (in review).

55. Demirel. H.A., S.K. Powers, K.L. Hamilton, H.K. Vincent, H. Naito, J.S. Coombes, and R.A. Shanely. The effect of endurance exercise on skeletal muscle HSP72 expression. *Medicine and Science in Sports and Exercise* 30(5): S204, 1998b.

56. Deshaies, R.J., B.D. Koch, M. Weiner-Washburne, E. Craig, and R. Schekman. A subfamily of stress proteins fascilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature (London)* 332: 800-805, 1988.

57. Dice, J.F., F. Agarraberes, M. Kirven-Brooks, L.J. Terlecky, and S.R. Terlecky. Heat shock 70-kDa proteins and lysosomal proteolysis. In: *The Biology of Heat Shock Proteins and Molecular Chaperones,* edited by R.I. Morimoto, A. Tissieres, and C. Georgopoulos. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1994, p. 137-152.

58. Dillman, W.H. and R. Mestril. Ischemia, infarction, and HSP70. In: *Heat Shock Proteins and the Cardiovascular System*, edited by A. Knowlton. Boston: Kluwer Academic Publishers, 1997, p. 1-24.

59. Dillman, W.H., H.B. Mehta, A. Barrieux, B.D. Guth, W.E. Neeley, and J. Ross. Ischemia of the dog heart induces the apperance of a cardiac mRNA coding for a protein with migration characteristics similar to heat shock/stress protein 71. *Circulation Research* 59: 110-114, 1986.

60. Dimitrov, N.V., C. Meyer, D.E. Ullrey, W. Chenoweth, A. Michelakis, W. Malone, C. Boone, and G. Fink. Bioavailability of beta-carotene in humans. *American Journal of Clinical Nutrition* 48(2): 298-304, 1988.

61. Donnelly, T., R. Sievers, F. Vissern, W. Welch, and C. Wolfe. Heat shock protein induction in rat hearts. A role for increased myocardial salvage after ischemia and reperfusion? *Circulation* 85: 769-778, 1992.

62. Downey, J. Free radicals and their involvement during long-term myocardial ischemia and reperfusion. *Annual Review of Physiology* 52: 487-504, 1990.

63. Ellis, R.J. Stress proteins as molecular chaperones. In: *Stress Proteins in Medicine,* edited by W. van Eden, and D.B. Young. New York: Marcel Decker Inc., 1996, p. 1-26.

64. Ellis, R.J. Do molecular chaperones have to be proteins? Biochemistry and. *Biophysics Research Communications* 238(3): 687-692, 1997.

65. Ellis, R.J. and S.M. van der Vies. Molecular chaperones. *Annual Review of Biochemistry* 60: 321-347, 1991.

66. Ernster, L. and G. Dallner. Biochemical, physiological and medical aspects of ubiquinone function. *Biochimica et Biophysica Acta* 1271(1): 195-204, 1995.

67. Fernandes, M., T. O'Brian, and J.T. Lis. Structure and regulation of heat shock gene promoters. In: *The Biology of Heat Shock Proteins and Molecular Chaperones,* edited by R.I. Morimoto, A. Tissieres, and C. Georgopoulos. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1994, p. 375-393.

68. Ferrara, N., P. Abete, G. Ambrosio, P. Landino, P. Caccese, P. Cirillo, A. Oradei, G.P. Littaru, M. Chiarello, and F. Rengo. Protective role of ubiquinones administration on acute cardiac oxidative stress. *Journal of Pharmacology and Experimental Therapy* 274:858-865, 1995.

69. Ferrari, R., O. Visioli, C. Guanieri, and C.M. Caldarera. Vitamin E and the heart: possible role as an antioxidant. *Acta Vitaminology Enzymology* 5: 11-22, 1983.

70. Fishbein, M., S. Meerbaum, J. Rit, U. Lando, K. Kanmatsuse, J.C. Mercier, E. Corday, and W. Ganz. Early phase acute myocardial infarct size quantification: Validation of the triphenyl tetrazolium chloride tissue enzyme staining technique. *American Heart Journal* 101: 593-600, 1981.

71. Flohe, L. and W. Gunzler. Assays of glutathione peroxidase. *Methods in Enzymology* 105: 114-121, 1984.

72. Frolov, V.A. and V.A. Kapustin. Effect of vitamin A and E on the contractile function of the heart in experimental myocardial infarction. *Kardiologica* 23: 93-95, 1983.

73. Frydman, J., E. Nimmesgern, K. Ohtuska, and F.U. Hartl. Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. *Nature* 370: 111-117, 1994.

74. Fuenmayor, A.J., A.M. Fuenmayor, T. Lopez, and D.M. Winterdaal. Vitamin E and ventricular fibrillation threshold in myocardial ischemia. *Japanese Circulation Journal* 53: 1229-1232, 1989.

75. Gaia, G., L. Comini, E. Pasini, G. Tomelleri, L. Agnoletti and R. Ferrari. Heat shock protein 72 in cardiac and skeletal muscles during hypertension. *Molecular and Cellular Biochemistry* 146(1): 1-6, 1995.

76. Garlick, P., M. Davies, D. Hearse, and T. Slater. Direct detection of free radicals in the reperfused rat heart using spin resonance spectroscopy. *Circulation Research* 61: 757-760, 1987.

77. Garner, T., J. Stewart, A. Casale, J. Downey, and D. Chambers. Reduction of myocardial ischemic injury with oxygen derived free radical scavengers. *Surgery* 94: 423-427, 1983.

78. Gauduel, Y. and M.A. Duvelleroy. Role of oxygen radicals in cardiac injury due to reoxygenation. *Journal of Molecular and Cellular Cardiology* 16: 459-470, 1984.

79. Gavva, S., A. Wiethoff, A.D. Sherry, C.R. Malloy, and R.S. Williams. Cardioprotective effects of 70-kDa heat shock protein in transgenic mice. *Proceedings of the National Academy of Science USA* 93: 2339-2342, 1996.

80. Geenen, D., A. Malhotra, D. Liang, and J. Scheur. Ventricular function and contractile proteins in the infarcted overloaded rat heart. *Journal of Cardiovascular Research* 25: 330-336, 1991.

81. Georgopoulos, C. and W.J. Welch. Role of the major heat shock proteins as molecular chaperones. *Annual Review of Cell Biology* 9: 601-634, 1993.

82. Gerner, E.W. and M.J. Scheider. Induced thermal resistance in HeLa cells. *Nature (London)* 256: 500-502, 1975.

83. Gething, M.J. and J.F. Sambrook. Protein folding in the cell. *Nature* 355: 35-45, 1992.

84. Gorman, A.M., B. Heavey, E. Creagh, T.G. Cotter, and A. Samali. Antioxidant-mediated inhibition of the heat shock response leads to apoptosis. *Federation of European Biochemical Societies Letters* 445(1): 98-102, 1999.

85. Green, E. Anatomy of the Rat. New York: Hafner Publishing Co., 1963, p. 50-62.

86. Guanieri, C., F. Flamigni, and C.R. Caldarera. Subcellular localization of alpha-tocopherol and its effect on RNA synthesis in perfused rabbit heart. *Italian Journal of Biochemistry* 29: 176-184, 1980.

87. Guanieri, C., R. Ferrari, O. Visioli, C.M. Caldarera, and W.G. Naylor. Effect of alpha-tocopherol on hypoxic-perfused and reoxygenated rabbit heart muscle. *Journal of Molecular and Cellular Cardiology* 10: 893-906, 1978.

88. Haddock, P.S., M.J. Shattock, and D.J. Hearse. Modulation of cardiac Na^+-K^+ pump current: role of protein and nonprotein sulfhydryl redox status. *American Journal of Physiology* 269: H297-H307, 1995

89. Halliwell, B. and S. Chirico. Lipid peroxidation: its mechanism, measurement, and significance. *American Journal of Clinical Nutrition* 57: 715S-725S, 1993.

90. Halliwell, B. and J. Gutteridge. Free Radicals in Biology and Medicine. New York: Oxford University Press, 1999.

91. Hamilton, K., S. Powers, T. Suguira, S. Lennon, S.J. Kim, and J. Mehta. Exercise-induced cardioprotection during ischemia-reperfusion in the absence of heat shock proteins. *American Journal of Physiology* (in review).

92. Hammond, G.I., Y.K. Lai, and C.L. Markert. Diverse forms of stress leas to new patterns of gene expression through a common and essential metabolic pathway. *Proceedings of the National Academy of Science USA* 79: 3485-3488, 1982.

93. Haramaki, N., H. Assadnazari, G. Zimmer, V. Schepkin, and L. Packer. The influence of vitamin E and dihydrolipoic acid on cardiac energy and glutathione status under hypoxia-reoxygenation. *Biochemistry and Molecular Biology International* 37: 591-597, 1995.

94. Haramaki, N., L. Packer, H. Assadnazari, and G. Zimmer. Cardiac recovery during post-ischemic reperfusion is improved by combination of vitamin E with dihydro-lipoic acid. *Biochemical and Biophysical Research Communications* 196: 1101-1107, 1993.

95. Haramaki, N., D.B. Stewart, S. Aggarwal, H. Ikeda, A.Z. Reznick, and L. Packer. Networking antioxidants in the isolated rat heart are selectively depleted by ischemia-reperfusion. *Free Radical Biology and Medicine* 25(3): 329-339, 1998.

96. Harnois, C., J. Samson, M. Malenfant, and A. Rousseau. Canthaxanthin retinopathy. Anatomic and functional reversibility. *Archives in Ophthalmology* 107(4): 538-540, 1989.

97. Hartman, D. and M.J. Gething. Normal protein folding machinery. *EXS* 77: 3-24, 1996.

98. Heads, R.J., D.S. Latchman, and D.M. Yellon. Stable high level expression of a transfected human hsp70 gene protects a heart-derived muscle cell line against thermal stress. *Journal of Molecular and Cellular Cardiology* 26(6): 695-699, 1994.

99. Hein, S., T. Sceffold, and J. Schaper. Ischemia induces early changes to cytoskeletal and contractile proteins in diseased human myocardium. *Journal of Thoracic and Cardiovascular Surgery* 110: 89-98, 1995.

100. Hellsten, Y. The role of xanthine oxidase in exercise, In: *Exercise and Oxygen Toxicity*, edited by C.K. Sen, L. Packer, and O. Hanninen. Amsterdam: Elsevier Science, 1995, p. 211-234.

101. Herendeen, S.L., R.A. Van Bogelen, and F.C. Neidhardt. Levels of major proteins of Escherichia coli during growth at different temperatures. *Journal of Bacteriology* 132: 255-258, 1979.

102. Hermes-Lima, M., W. Willmore, and K. Storey. Quantification of lipid peroxidation in tissue extracts based on Fe(III) xylenol orange formation. *Free Radical Biology and Medicine* 19: 271-280, 1995.

103. Heywood, R., A.K. Palmer, R.L. Gregson, and H. Hummler. The toxicity of beta-carotene. *Toxicology*. 36(2-3): 91-100, 1985.

104. Hightower, L.E. Cultured animal cells exposed to amino acid analogues or puromycin rapidly synthesize several polypeptides. *Journal of Cellular Physiology* 102: 407-427, 1980.

105. Horwitz, L.D., P.V. Fennessey, R.H. Shikes, and Y. Kong. Marked reduction in myocardial infarct size due to prolonged infusion of an antioxidant during reperfusion. *Circulation* 89(4): 1792-801, 1994.

106. Hutter J.J., R. Mestril, E.K. Tam, R.E Sievers, W.H. Dillmann and C.L Wolfe. Overexpression of heat shock protein 72 in transgenic mice decreases infarct size in vivo. *Circulation* 94: 1408-1411, 1996.

107. Hutter M.M., R.E. Sievers, V. Barbosa and C.L. Wolfe. Heat-shock protein induction in rat hearts. A direct correlation between the amount of heat-shock protein induced and the degree of myocardial protection. *Circulation* 89: 355-360, 1994.

108. Ibrahim, W., U-S. Lee, H-C. Yen, D.K. St. Clair, and C.K. Chow. Antioxidant and oxidative status in tissues of manganese superoxide dismutase transgenic mice. *Free Radical Biology and Medicine* 28(3): 397-402, 2000.

109. Isoyama S. Changes in heat shock proteins in cardiac hypertrophy. In: *Heat shock proteins and the cardiovascular system*, edited by A. Knowlton. Boston: Kluwer Academic Publishers, 1997, p. 83-98.

110. Janero, D. Therapeutic potential of vitamin E against myocardial ischemic-reperfusion injury. *Free Radical Biology and Medicine* 10: 315-324, 1991.

111. Ji, L.L. Exercise and oxidative stress: role of the cellular antioxidant systems. *Exercise and Sport Science Reviews* 23: 135-166, 1995.

112. Ji, L., D. Dillon, and E. Wu. Alterations of antioxidant enzymes with aging in rat skeletal muscle and liver. *American Journal of Physiology* 258: R918-R923, 1990.

113. Ji, L.L., R. Fu, and E.W. Mitchell. Glutathione and antioxidant enzymes in skeletal muscle: effects of fiber type and exercise intensity. *Journal of Applied Physiology* 73(5): 1854-1859, 1992.

114. Ji, L., R. Fu, E. Mitchell, M. Griffiths, T. Waldrop, and H. Swartz. Cardiac hypertrophy alters myocardial response to I-R in vivo. *Acta Physiologica Scandinavica* 151: 270-290, 1994.

115. Jocelyn, P.C. Spectrophotometric assay of thiols. *Methods in Enzymology* 143: 44-67, 1987.

116. Kagan, V., A. Shvedova, E. Serbinova, S. Khan, C. Swanson, R. Powell, and L. Packer. Dihydrolipoic acid-a universial antioxidant both in the membrane and in the aqueous phase. *Biochemical Pharmacology* 44: 1637-1649, 1992.

117. Kagan, V., E. Serbinova, and L. Packer. Antioxidant effects of ubiquinones in microsomes and mitochondria are mediated by tocopherol recycling. *Biochemical and Biophysical Research Communication* 169: 851-857, 1990.

118. Kagan, V.E., E.A.K. Serbinova, T. Forte, G. Scita, and L. Packer. Recycling of vitamin E in human low density lipoproteins. *Journal of Lipid Research* 33: 385-397, 1992.

119. Kappus, H. and A.T. Diplock. Tolerance and safety of vitamin E: a toxological position report. *Free Radical Biology and Medicine* 13: 55-74, 1992.

120. Kawasaki, T., K. Sugino, and S. Marubayashi. The role of cellular antioxidants and administered coenzyme Q_{10} in oxidative cellular damage-experimental liver ischemia and endotoxemia. In: *Biochemical and Clinical Aspects of Coenzyme Q*, edited by K. Folkers, and Y. Yamamura. Amsterdam: Elsevier Science, 1995, p. 145-155.

121. Keyse, S.M., L.A. Applegate, Y. Tromvoukis, and R.M. Tyrell. Oxidant stress leads to transcriptional activation of the human heme oxygenase gene in cultured skin fibroblast. *Molecular and Cell Biology* 10: 4967-4969, 1990.

122. Keyse, S. M. and R.M. Tyrell. Heme oxygenase is the major 32 kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide and sodium arsenite. *Proceedings of the National Academy of Science USA* 86: 99-103, 1989.

123. Kim, D.H., H. Quyang, and G.C. Li. Heat shock protein hsp70 accelerates the recovery of heat-shocked mammalian cells through its modulation of heat shock transcription factor HSF1. *Proceedings of the National Academy of Science USA* 92: 2126-2130, 1995.

124. Klein, H., S. Pich, S. Lindert, K. Nebendahl, P. Niedmann, and H. Kreuzer. Combined treatment with vitamins E and C in experimental myocardial infarction in pigs. *American Heart Journal* 118: 667-673, 1989.

125. Knowlton, A.A., P. Brecher, and C.S. Apstein. Rapid expression of heat shock protein in the rabbit after brief cardiac ischemia. *Journal of Clinical Investigation* 87: 139-147, 1991.

126. Knowlton, A. An overview of the heat shock proteins, their regulation, and function. In: *Heat Shock Proteins and the Cardiovascular System*, edited by A. Knowlton. Boston: Kluwer Academic Publishers, 1997, p. 1-24.

127. Knudsen C.A., A.L. Tappel, and J.A. North. Multiple antioxidants protect against heme protein and lipid oxidation in kidney tissue. *Free Radical Biology and Medicine* 20(2): 165-173, 1996.

128. Kotegawa, M., M. Sugiyama, T. Shoji, H. Haramaki, and R. Ogura. Effect of alpha-tocopherol on high energy phosphate metabolite levels in rat heart by 31P-NMR using a Langerdorff perfusion technique. *Journal of Molecular and Cellular Cardiology* 25: 1067-1074, 1993.

129. Kregel, K.C. and P.L. Moseley. Differential effects of exercise and heat stress on liver. HSP70 accumulation with aging. *Journal of Applied Physiology* 80: 547-551, 1996.

130. Kukreja, R.C., M.C. Kontos, K.E. Loesser, S.K. Batra, Y-Z. Qian, C.J. Gbur, S.A. Naseem, R.L. Jesse, and M.L. Hess. Oxidant stress increases heat shock protein 70 mRNA in isolated perfused rat heart. *American Journal of Physiology* 267: H2213-H2219, 1994.

131. Lawler, J., D. Criswell, and S. Powers. Oxygen cost of treadmill running in 24-month old Fischer-344 rats. *Medicine and Science in Sports and Exercise* 25: 1259-1264, 1993.

132. Leeuwenburgh, C. and L.L. Ji. Alteration of glutathione and antioxidant status with exercise in unfed and refed rats. *Journal of Nutrition* 126(7): 1833-1843, 1996.

133. Leeuwenburgh, C. and L.L Ji. Glutathione and glutathione ethyl ester supplementation of mice alter glutathione homeostasis during exercise. *Journal of Nutrition* 128: 2420-2426, 1998.

134. Leeuwenburgh, C., J. Hollander, S. Leichtweis, M. Griffiths, M. Gore, and L.L. Ji. Adaptations of glutathione antioxidant system to endurance training are tissue and muscle fiber specific. *American Journal of Physiology* 272(1 Pt 2): R363-369, 1997.

135. Leeuwenburgh, C., S. Leichtweis, J. Hollander, R. Fiebig, M. Gore, and L.L. Ji. Effect of acute exercise on glutathione deficient heart. *Molecular and Cellular Biochemistry* 156: 17-24, 1996.

136. Lehr, H.A., M.D. Menger, and K. Messmer. Impact of leukocyte adhesion on myocardial ischemia/reperfusion injury: conceivable mechanisms and proven facts. *Journal of Laboratory and Clinical Medicine* 121: 539-545, 1993.

137. Leichtweis, S., C. Leeuwenburgh, R. Chandwaney, and D. Parmelee. Ischemia-reperfusion induced alterations of mitochondrial function in hypertrophied rat heart. *Acta Physiologica Scandinavica* 156: 51-60, 1996.

138. Lesnefsky, E.J. Reduction of infarct size by cell-permeable oxygen metabolite scavengers. *Free Radical Biology and Medicine* 12(5): 429-46, 1992.

139. Levinson, W., H. Oppermann, and J. Jackson. Transition series metals and sulfhydryl reagents induce the synthesis of four proteins in eukaryotic cells. *Biochimica et Biophysica Acta* 606: 170-180, 1980.

140. Li, G.C. and Z. Werb, Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. *Proceedings of the National Academy of Science USA* 79: 3218-3222, 1982.

141. Li, G.C., L.G. Li, Y.K. Liu, J.Y. Mak, L.L. Chen, and W.M. Lee. Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding gene. *Proceedings of the National Academy of Science USA* 88: 1681-1685, 1991.

142. Lindquist, S. The heat shock response. *Annual Review of Biochemistry* 55: 1151-1191, 1986.

143. Lindquist, S. and E.A. Craig. The heat-shock proteins. *Annual Review of Genetics* 22: 631-677, 1988.

144. Liu, R.Y. and G.C. Li. Expression of human hsp70 in rat fibroblasts enhances cell survival and facilitates recovery from translational and transcriptional inhibition following heat shock. *Cancer Research* 52: 3997-3673, 1992.

145. Lloyd. R.V., P.M. Hanna, and R.P. Mason. The origin of the hydroxyl radical oxygen in the fenton reaction. *Free Radical Biology and Medicine* 22: 885-888, 1997.

146. Locke, M. The cellular stress response to exercise: role of stress proteins. *Exercise and Sport Science Reviews* 25: 105-136, 1997.

147. Locke, M. and E.G. Noble. Stress proteins: The exercise response. *Canadian Journal of Applied Physiology* 20(2): 155-167, 1995.

148. Locke, M., E.G. Noble, and B.G. Atkinson. Exercising mammals synthesize stress proteins. *American Journal of Physiology* 258: C723-C729, 1990.

149. Locke, M., E.G. Noble, R.M. Tanguay, M. Field, R.S.E. Ianuzzo, and C.D. Ianuzzo. Activation of heat-shock transcription factor in rat heart after heat shock and exercise. *American Journal of Physiology* 268: C1387-C1394. 1995.

150. Locke, M., R.M. Tanguay, R.E. Klabunde, and C.D. Ianuzzo. Enhanced postischemic myocardial recovery following exercise induction of HSP 72. *American Journal of Physiology* 269: H320-H325, 1995.

151. Lonnrot, K., J.P. Tolvanen, I. Porsti, T. Ahola, A. Hervonen, and H. Alho. Coenzyme Q10 supplementation and recovery from ischemia in senescent rat myocardium. *Life Sciences* 64(5): 315-323, 1999.

152. Losowsky, M.S., J. Kelleher, and B.E. Walker. Intake and absorption of tocopherol. *Annals of the New York Academy of Sciences* 202: 212-222, 1972.

153. Lotito, S.B. and C.G. Fraga. (+)-Catechin prevents human plasma oxidation. *Free Radicals in Biology and Medicine* 24(3): 435-441, 1998.

154. Machlin, L.J. and E. Gabriel. Kinetics of tissue alpha-tocopherol uptake and depletion following administration of high levels of vitamin E. *Annals of the New York Academy of Sciences* 393: 48-60, 1982.

155. Maellaro, E., B. Del Bello, L. Sugherini, A. Pompella, A.F. Casini, and M. Comporti. Protection by ascorbic acid against oxidative injury of isolated hepatocytes. *Xenobiotica* 24(3): 281-289, 1994.

156. Manach, C., C. Morand, O. Texier, M.L. Favier, G. Agullo, C. Demigne, F. Regerat, and C. Remesy. Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. *Journal of Nutrition* 125(7): 1911-1922, 1995.

157. Marber, M., D.S. Latchman, J.M. Walker, and D.M. Yellon. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 88: 1264-1272, 1993.

158. Massey, K.D. and K.P. Burton. Alpha-tocopherol attenuates myocardial membrane-related alterations resulting from ischemia and reperfusion. *American Journal of Physiology* 256: H1192-H1199, 1989.

159. Massoro, E. Mortality and growth characteristics of rat strains commonly used in aging research. *Experimental Aging Research* 6: 219-233, 1980.

160. Maulik, N., H.S. Sharma, and D.K. Das. Induction of heme oxygenase gene expression during the reperfusion of ischemic rat myocardium. *Journal of Molecular and Cellular Cardiology* 28: 1261-1270, 1996.

161. Maulik, N. T. Yoshida, R.M. Engelman, D. Bagchi, H. Otani, and D.K. Das. Dietary coenzyme Q_{10} supplement renders swine hearts resistant to ischemia-reperfusion injury. *American Journal of Physiology Heart and Circulatory Physiology* 278: H1084-H1090, 2000.

162. Mayne, S.T. and R.S. Parker. Antioxidant activity of dietary canthaxanthin. *Nutrition and Cancer* 12(3): 225-236, 1989.

163. McCord, J. Oxygen-derived free radicals in post-ischemic tissue injury. *New England Journal of Medicine* 312: 159-163, 1985.

164. McCord, J. and I. Fridovich. Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry* 244(22): 6049-6055, 1969.

165. McDonald, M.C., K. Zacharowski, J. Bowes, S. Cuzzocrea, and C. Thiemermann. Tempol reduces infarct size in rodent models of regional myocardial ischemia and reperfusion. *Free Radical Biology and Medicine* 27(5-6): 493-503, 1999.

166. Meerson, F.Z. and E.E. Ustinova. Prevention of stress injury to the heart and its hypoxic contracture by using the natural antioxidant alpha-tocopherol. *Kardiologicia* 22: 89-94, 1982.

167. Mehta, H.B., B.K. Popovich, and W.H. Dillmann. Ischemia induces changes in the level of mRNAs coding for stress protein 71 and creatine kinase M. *Circulation Research* 63: 512-517, 1988.

168. Meister A. and M.E. Anderson. Glutathione. *Annual Review of Biochemistry* 52: 711-760, 1983.

169. Mellors, A. and A.L. Tappel. The inhibition of mitochondrial peroxidation by ubiquinone and ubiquinol. *Journal of Biological Chemistry* 241(19): 4353-4356, 1966.

170. Mestril, R., S.H. Chi, M.R. Sayen, and W.H. Dillmann. Isolation of a novel inducable rat heat shock protein (HSP70) gene and its expression during ischemia-hypoxia and heat shock. *Biochemistry Journal* 298: 561-569, 1994.

171. Mestril, R., S.H. Chi, M.R. Sayen, K. O'Reilly, and W.H. Dillmann. Expression of inducable stress protein 70 in rat heart myogenic cells confers protection against stimulated ischemia induced injury. *Journal of Clinical Investigation* 93: 759-767, 1994.

172. Mestril, R. and W.H. Dillmann. Heat shock proteins and protection against myocardial ischemia. *Journal of Molecular and Cellular Cardiology* 27: 45-52, 1995.

173. Miao, B., J. Davis, and E.C. Craig. The Hsp70 family-an overview. In: *Guidebook to Molecular Chaperones and Protein-Folding Catalysts,* edited by M.J. Gething. Istanbul: Oxford University Press, 1998, p. 3-12.

174. Miller N.J., J. Sampson, L.P. Candeias, P.M. Bramley, and C.A. Rice-Evans. Antioxidant activities of carotenes and xanthophylls. *Federation of European Biochemical Societies Letters* 384(3): 240-242, 1996.

175. Morimoto, R.I. Cells in stress: transcriptional activation of heat shock genes. *Science* 259: 1409-1410, 1993.

176. Morimoto, R.I., D.A. Jurivich, and P.E. Kroeger. The regulation of heat shock gene expression by a family of heat shock factors. In: *The Biology of Heat Shock Proteins and Molecular Chaperones,* edited by R.I. Morimoto, A. Tissieres, and C. Georgopoulos. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1994, p. 417-455.

177. Morimoto, R.I., M.P. Kline, D.N. Bimston, and J.J. Cotto. The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. *Essays in Biochemistry* 32: 17-29, 1997.

178. Morimoto, R.I., K.D. Sarge, and K. Abravaya. Transcriptional regulation of heat shock genes. A paradigm for inducible genomic responses. *Journal of Biological Chemistry* 267: 21987-21990, 1992.

179. Munro, S. and H.R.B. Pelham. An Hsp70-like protein in the ER: identity with the 78kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* 46: 294-300, 1986.

180. Nakagawa, Y., I.A. Cotgreave, and P. Moldeus. Relationships ascorbic acid and alpha tocopherol during diquat-induced redox cycling in isolated rat hepatocytes. *Biochemical Pharmacology* 42: 883-888, 1991.

181. Neufer, P.D. and I.J. Benjamin, Differential expression of αB-Crystalin and Hsp27 in skeletal muscle during continuous contractile activity. *Journal of Biological Chemistry* 271(39): 24089-24095, 1996.

182. Newaz, M.A. and N.N. Nawal. Effect of alpha-tocopherol on lipid peroxidation and total antioxidant status in spontaneously hypertensive rats. *American Journal of Hypertension* 11(12): 1480-1485, 1998.

183. Newaz M.A. and N.N. Nawal. Effect of gamma-tocotrienol on blood pressure, lipid peroxidation and total antioxidant status in spontaneously hypertensive rats (SHR). *Clinical and Experimental Hypertension* 21(8): 1297-1313, 1999.

184. Nishizawa, J., A. Nakai, K. Matsuda, M. Komeda, T. Ban, and K. Nagata. Reactive oxygen species play an important role in the activation of heat shock factor 1 in ischemic-reperfused heart. *Circulation* 99(7): 934-941, 1999.

185. North, J.A. and A.L. Tappel. Measuring the oxidation of heme compounds in heart homogenates from rats supplemented with dietary antioxidants. *Free Radical Biology and Medicine* 22(1-2): 175-184, 1997.

186. Oscai, L., P. Mole, and J. Holloszy. Effects of exercise on cardiac weight and mitochondria in male and female rats. *American Journal of Physiology* 220: 1944-1948, 1971.

187. Packer L. Protective role of vitamin E in biological systems. *American Journal of Clinical Nutrition* 53(4 Suppl): 1050S-1055S, 1991.

188. Packer, I.E., T.F. Slater, and R.L. Willson. Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* 278: 737-738, 1979.

189. Palozza, P., S. Moualla, and N.I. Krinsky. Effects of beta-carotene and alpha-tocopherol on radical-initiated peroxidation of microsomes. *Free Radical Biology and Medicine* 13(2): 127-136, 1992.

190. Paradies, G., G. Petrosillo, M. Pistolese, N. DiVenosa, D. Serena, and F.M. Ruggiero. Lipid peroxidation and alterations to oxidative metabolism in mitochondria isolated from rat heart subjected to ischemia and reperfusion. *Free Radical Biology and Medicine* 27(1/2): 42-50, 1999.

191. Park, Y., S. Kanekal, and J.P. Kehrer. Oxidative changes in hypoxic rat heart tissue. *American Journal of Physiology* 260: H1395-H1405, 1991.

192. Parker, C.S. and J. Topol. A Dorsophila RNA polymerase II transcription factor binds to the regulatory site of an hsp70 gene. *Cell* 37: 273-283, 1984.

193. Pelham, H.R.B. A regulatory upstream promoter element in the *Drosophila* hsp 70 heat shock gene. *Cell* 30: 517-528, 1982.

194. Petty, M., J. Grisar, and W. De Jong. Protective effects of an alpha-tocopherol analogue against myocardial reperfusion injury in rats. *European Journal of Pharmacology* 210: 85-90. 1992.

195. Plumier, J.C., B.M. Ross, R.W. Currie, and C.E. Angelidis. Transgenic mice expressing the human heat shock protein 70 have improved postischemic myocardial recovery. *Journal of Clinical Investigation* 95: 1854-1860, 1995.

196. Poston, J.M. and G.L. Parenteau. Biochemical effects of ischemia on isolated, perfused rat heart tissues. *Archives of Biochemistry and Biophysics* 15: 35-41, 1992.

197. Powers, S., D. Criswell, J. Lawler, D. Martin, F. Lieu, and L.L. Ji. Rigorous exercise training increases superoxide dismutase activity in the ventricular myocardium. *American Journal of Physiology* 265: H2094-H2098, 1993.

198. Powers, S.K., H.A. Demirel, H.K. Vincent, J.S. Coombes, H. Naito, K.L. Hamilton, R.A. Shanely, and J. Jessup. Exercise training improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. *American Journal of Physiology* 275: R1468-77, 1998.

199. Powers S.K. and K.L. Hamilton. Antioxidants and exercise. *Clinics in Sports Medicine* 18(3): 525-536, 1999.

200. Pratt, W.B., L.C. Scherrer, K.A. Hutchinson, and F.C. Dalman. A model of glucocorticoid receptor unfolding and stabilization by a heat shock protein complex. *Journal of Steroid Biochemistry* 41: 223-229, 1992.

201. Pyke, D.D. and A.C. Chan. Effects of vitamin E on prostacyclin release and lipid composition of the ischemic rat heart. *Archives of Biochemistry and Biophysics* 277: 429-433, 1990.

202. Reed, D.J., J.R. Babson, P.W. Beatty, A.E. Brodie, W.W. Ellis, and D.W. Potter. High-performance liquid chromotography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Analytical Biochemistry* 106: 55-62, 1980.

203. Reeves, P.G., F.H. Nielsen, and G.C. Fahey. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *Journal of Nutrition* 123(11): 1939-1951, 1993.

204. Reid, M. Reactive oxygen species and nitric oxide in skeletal muscle. *News in Physiological Sciences* 11: 114-119, 1996.

205. Reid M.B., D.S. Stokic, S.M. Koch, F.A. Khawli, and A.A. Leis. N-acetylcysteine inhibits muscle fatigue in humans. *Journal of Clinical Investigation* 94(6): 2468-2474, 1994.

206. Report of the US Preventative Services Task Force: Guide to Clinical Preventative Services: An Assessment of the Effectiveness of 169 Interventions. Baltimore: Williams and Wilkins, 1989, p. 3.

207. Reznick, A. and L. Packer. Oxidative damage to proteins: spectrophotometric method for oxidatively modified proteins. *Methods in Enzymology* 223: 357-362, 1994.

208. Riabowol, K., L.A. Mizzen, and W.J. Welch. Heat shock induced lethality in cells microinjected with antibodies specific for hsp 70. *Science* 242: 433-436, 1988.

209. Ritossa, F.M. A new puffing pattern induces by a temperature shock and DNP in Drosophilia. *Experientia (Basel)* 18: 571-573, 1962.

210. Rojas, C., S. Cadenas, R. Perez-Campo, M. Lopez-Torres, and G. Barja. Effect of vitamin C on antioxidants, lipid peroxidation, and GSH system in the normal guinea pig heart. *Journal of Nutrition Science Vitaminology (Tokyo)* 40(5): 411-420, 1994.

211. Romaschin, A.D., I. Rebeyka, G.J. Wilson, and D.A.G. Mickle. Conjugated dienes in ischemic and reperfused myocardium: an *in vivo* chemical signature of oxygen free radical mediated injury. *Journal of Molecular and Cellular Cardiology* 19: 289-302, 1987.

212. Romaschin, A.D., G.J. Wilson, U. Thomas, D.A. Feitler, L. Tumiati, and D.A.G. Mickle. Subcellular distribution of peroxidized lipids in myocardial reperfusion injury. *American Journal of Physiology* 259: H116-H123, 1990.

213. Rousseau E.J., A.J. Davison, and B. Dunn. Protection by beta-carotene and related compounds against oxygen-mediated cytotoxicity and genotoxicity: implications for carcinogenesis and anticarcinogenesis. *Free Radical Biology and Medicine* 13(4): 407-433, 1992.

214. Saija, A., M. Scalese, M. Lanza, D. Marzullo, F. Bonina, and F. Castelli. Flavonoids as antioxidant agents: importance of their interaction with biomembranes. *Free Radical Biology and Medicine* 19(4): 481-486, 1995.

215. Salah, N., N.J. Miller, G. Paganga, L. Tijburg, G.P. Bolwell, and C. Rice-Evans. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Archives in Biochemistry and Biophysics* 322(2): 339-346, 1995.

216. Salo, D.C, C.M. Donovan, and K.J. Davies. HSP70 and other possible heat shock or oxidative stress proteins are induced in skeletal muscle, heart, and liver during exercise. *Free Radical Biology and Medicine* 11: 239-246, 1991.

217. Sarge, K.D., S.P. Murphy, and R.I. Morimoto. Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. *Molecular and Cell Biology* 13: 1392-1407, 1993.

218. Scholich, H., M. Murphy, and H. Sies. Antioxidant activity of dihydrolipoate against microsomal lipid peroxidation and its dependence on alpha-tocopherol. *Biochimca et Biophysica Acta* 1001: 256-261, 1989.

219. Serbinova, E., S. Khwaja, A.Z. Reznick, and L. Packer. Thioctic acid protects against ischemia-reperfusion injury in the isolated perfused Langerdorff heart. *Free Radical Research Communications* 17: 49-58, 1992.

220. Sethi, R., N. Takeda, M. Nagano, and N.S. Dhalla. Beneficial effects of vitamin E treatment in acute myocardial infarction. *Journal of Cardiovascular and Pharmacological Therapy* 5: 51-58, 2000.

221. Sharma, H.S. and J. Stahl. Role of small heat shock proteins in the cardiovascular system. In: *Heat Shock Proteins and the Cardiovascular System*, edited by A. Knowlton. Boston: Kluwer Academic Publishers, 1997, p. 127-158.

222. Shindoh C., A. DiMarco, A. Thomas, P. Manubay, and G. Supinski. Effect of N-acetylcysteine on diaphragm fatigue. *Journal of Applied Physiology* 68(5): 2107-2113, 1990.

223. Sichel, G., C. Corsaro, M. Scalia, A.J. Di Bilio, and R.P. Bonomo. In vitro scavenger activity of some flavonoids and melanins against O2-(˙). *Free Radical Biology and Medicine* 11(1): 1-8, 1991.

224. Skidmore, R.J., A. Gutierrez, V. Guerriero, and K.C. Kregel. HSP70 induction during exercise and heat stress in rats: role of internal temperature. *American Journal of Physiology* 268: R92-R97, 1995.

225. Subjeck, J.R., T. Shyy, J. Shen, and R.J. Johnson. Association between mammalian 110.000 dalton heat shock proteins and nucleoli. *Journal of Cell Biology* 97: 1389-1398, 1983.

226. Sugiyama, S., M. Kitazawa, and T. Qzawa. Protective effect of Coenzyme Q_{10} . *Experentia* 36: 1002-1003, 1980.

227. Sullivan, V., S. Powers, D. Criswell, N. Tumer, J. LaRochelle, and D. Lowenthal. Myosin heavy chain composition in young and old rat skeletal muscle: effects of endurance exercise. *Journal of Applied Physiology* 78: 2115-2120, 1995.

228. Suzuki, Y.J., M. Tsuchiya, and L. Packer. Thioctic acid and dihydrolipoic acid are novel antioxidants which interact with reactive oxygen species. *Free Radical Research Communications* 13: 255-263, 1991.

229. Swies, J., E.K.I. Omogbai, and G.M. Smith. Occlusion and reperfusion induced arrhythmias in rats: involvement of platelets and effects of calcium antagonists. *Journal of Cardiovascular Pharmacology* 15: 816-825, 1990.

230. Szarkowska, L. The restoration of DNPH oxidase activity in Coenzyme Q (ubiquinone). *Archives in Biochemistry and Biophysics* 113: 519-525, 1966.

231. Tanaka, M., H. Fujiwara, K. Yamasaki, M. Miyamae, R. Yokota, K. Hasegawa, T. Fujiwara, and S. Sasayama. Ischemic preconditioning elevates cardiac stress protein but does not limit infarct size 24 or 48 h later in rabbits. *American Journal of Physiology* 36: H1476-1482, 1995.

232. Tanaka, M., V.J. Richard, C.E. Murry, R.B. Jennings, and K.A. Reimer. SOD plus CAT therapy delays neither cell death nor the loss of the TTC reaction in experimental myocardial infarction in dogs. *Journal of Molecular and Cellular Cardiology* 25(4): 367-78, 1993.

233. Tappel, A.L. Lipid peroxidation damage to cell components. *Federal Proceedings* 32: 1870-1874, 1972.

234. Tate, C.A., G.E. Taffet, E.K. Hudson, S.L. Blaylock, R.P. McBride, and L.H. Michael. Enhanced calcium uptake of cardiac sarcoplasmic reticulum in exercise-trained old rats. *American Journal of Physiology* 258(2 Pt 2): H431-435, 1990.

235. Taylor R.P., M.B. Harris, and J.W. Starnes. Acute exercise can improve cardioprotection without increasing heat shock protein content. *American Journal of Physiology* 276 (3 Pt 2): H1098-H1102, 1999.

236. Theroux P. Protection of the myocardial cell during ischemia. *American Journal of Cardiology* 83(10A): 3G-9G, 1999.

237. Thomas, S.R., J. Neuzil, and R. Stocker. Cosupplementation with coenzyme Q prevents the prooxidant effect of alpha-tocopherol and increases the resistance of LDL to transition metal-dependent oxidation initiation. *Arteriosclerosis and Thrombosis Vascular Biology* 16(5): 687-696, 1996.

238. Tissieres, A., H.K. Mitchell, and U.M. Tracy. Protein synthesis in salivary glands of *Drosophilia melanogaster*. *Journal of Molecular Biology* 84: 389-398, 1974.

239. Ushakova, T., H. Melkonyan, L. Nikonova, N. Mudrik, V. Gogvadze, A. Zhukova, A.I. Gaziev, and R. Bradbury. The effect of dietary supplements on gene expression in mice tissues. *Free Radical Biology and Medicine* 20(3): 279-284, 1996.

240. Venditti, P. P. Masullo, S. Di Meo, and C. Agnisola. Protection against ischemia-reperfusion induced oxidative stress by vitamin E treatment. *Archives of Physiological Biochemistry* 107: 27-34, 1999.

241. Vile, G.F., S. Basu-Modak, C. Waltner, and R.M.Tyrell. Heme oxygenase I mediates an adaptive response to oxidative stress in human skin fibroblasts. *Proceedings of the National Academy of Science USA* 91: 2607-2610, 1994.

242. Villari, B., G. Ambrosio, P. Golino, M. Ragni, A. Focaccio, I. Tritto, M. Salvatore, and M. Chiariello. The effects of calcium channel antagonist treatment and oxygen radical scavenging on infarct size and the no-reflow phenomenon in reperfused hearts. *American Heart Journal* 125(1): 11-23, 1993.

243. Walker, D.M., E. Pasini, S. Kucukoglu, M.S. Marber, E. Iliodromitis, R. Ferrari and D.M. Yellon. Heat stress limits infarct size in the isolated perfused rabbit heart. *Cardiovascular Research* 27: 962-967, 1993.

244. Warner, B.B., L. Stuart, S. Gebb, and J.R. Wispe. Redox regulation of manganese superoxide dismutase. *American Journal of Physiology* 271(15): L150-L158, 1996.

245. Watanabe, B.I., S. Premaratne, W. Limm, M.M. Mugiishi, and J.J. McNamara. High- and low-dose SOD plus CAT does not reduce myocardial infarct size in a subhuman primate model. *American Heart Journal* 126(4): 840-846, 1993.

246. Wayner, D.D.M, G.W. Burton, and K.U. Ingold. The antioxidant efficiency of vitamin C is concentration-dependent. *Biochimica et Biophysica Acta* 884: 119-123, 1986.

247. Weich, H., J. Buchner, R. Zimmermann, and U. Jakob. Hsp 90 chaperones protein folding in vitro. *Nature* 358: 169-170, 1992.

248. Welch W.J. The mammalian stress response: cell physiology, and biochemistry of stress proteins. In: *Stress Proteins in Biology and Medicine,* edited by R.I. Morimoto, A. Tissieres, and C. Georgopopulus. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1990, p. 223-227.

249. Welch, W.J. Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiological Reviews* 72: 1063-1081, 1992.

250. Welch, W.J., H.S. Kang, R.P. Beckmann, and L.A. Mizzen. Response of mammalian cells to metabolic stress; changes in cell physiology and structure/function of stress proteins. *Current Topics in Microbiology and Immunology* 167: 31-55, 1991.

251. Werns, S.W., J.C. Fantone, A. Ventura, and B.R. Lucchesi. Myocardial glutathione depletion impairs recovery of isolated blood-perfused hearts after global ischaemia. *Journal of Molecular and Cellular Cardiology* 24(11): 1215-1220, 1992.

252. Williams, R.S., J.A. Thomas, M. Fina, Z. German and I.J.Benjamin. Human heat shock protein 70 (HSP70) protects murine cells from injury during metabolic stress. *Journal Clinical Investigation* 92: 503-508, 1993.

253. Woodall, A.A., G. Britton, and M.J. Jackson. Dietary supplementation with carotenoids: effects on alpha-tocopherol levels and susceptibility of tissues to oxidative stress. *British Journal of Nutrition* 76(2): 307-317, 1996.

254. Woodall, A.A., G. Britton, and M.J. Jackson. Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals:

relationship between carotenoid structure and protective ability. *Biochimica et Biophysica Acta* 1336(3): 575-586, 1997.

255. Wu, C. Two protein-binding sites in chromatin implicated in the activation of heat shock genes. *Nature* 309: 229-234, 1984.

256. Wu, C. Heat shock transcription factors: structure and regulation. *Annual Review of Cell Development and Biology* 11: 441-469, 1995.

257. Xiao, H. and J.T. Lis. Germline transformation used to define key features of heat shock response elements. *Science* 239: 1139-1142, 1988.

258. Yamamoto, K., M. Takahashi, and E. Niki. Role of iron and ascorbic acid in the oxidation of methyl linoleate micelles. *Chemical Letters* 116: 1149-1152, 1987.

259. Yamashita, N., S. Hoshida, N. Taniguchi, T. Kuzuya, and M. Hori. Whole-body hyperthermia provides biphasic protection against ischemia/reperfusion injury in the rat. *Circulation* 98:1414-1421, 1998.

260. Yamashita, N., S. Hoshida, K. Otsu, M. Asahi, T. Kuzuya, and M. Hori. Exercise provides direct biphasic cardioprotection via manganese superoxide dismutase activation. *Journal Experimental Medicine* 189(11): 1699-706, 1999.

261. Yan L.J., M.G. Traber, H. Kobuchi, S. Matsugo, H.J. Tritschler, and L. Packer. Efficacy of hypochlorous acid scavengers in the prevention of protein carbonyl formation. *Archives of Biochemistry and Biophysics* 327: 330-334, 1996.

262. Yoshida, T., G. Maulik, D. Bagchi, S.K. Dash, R.M. Engelman, and D.K. Das. Increased myocardial tolerance to ischemia-reperfusion injury by feeding pigs with coenzyme Q10. *Annals of the New York Academy of Sciences* 793: 414-418, 1996.

263. Yu, B. Cellular defenses against damage from reactive oxygen species. *Physiological Reviews* 74: 139-162, 1994.

264. Zhang, Y., F. Aberg, E.L. Appelkvist, G. Dallner and L. Ernster. Uptake of dietary coenzyme Q supplement is limited in rats. *Journal of Nutrition* 125(3): 446-453, 1995.

265. Zweier, J. Measurement of superoxide-derived free radicals in the reperfused heart. *Journal of Biological Chemistry* 263: 1353-1357, 1988.

266. Zweier, J., J. Flaherty, and M. Weisfeldt. Direct measurement of free radical generation following perfusion of ischemic myocardium. *Proceedings of the National Academy of Science USA* 84: 1404-1407, 1987.

BIOGRAPHICAL SKETCH

Karyn L. Hamilton was born in Wyoming and achieved her undergraduate degree in human nutrition from Montana State University in 1989. She worked as a registered dietition at the University of California, San Francisco and the University of Washington Medical Centers in Seattle until 1994 when she returned to Montana State University to complete the Master of Science degree in exercise physiology. Following completion of her M.S. in 1996, she began her doctoral program at the University of Florida and was awarded the Grinter Fellowship which, in part, supported her graduate studies. Following graduation, Karyn will begin a postdoctoral fellowship in the division of cardiology at Baylor College of Medicine in Houston, Texas.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Scott K. Powers, Chair
Professor of Exercise and Sport
Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Stephen L. Dodd
Associate Professor of Exercise and
Sport Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Christiaan Leeuwenburgh
Assistant Professor of Exercise and
Sport Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ralph Dawson
Associate Professor of
Pharmacodynamics

This dissertation was submitted to the Graduate Faculty of the College of Health and Human Performance and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 2000

Dean, College of Health and Human Performance

Dean, Graduate School